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HEPARIN PHARMACOKINETICS AND PHARMACODYNAMICS IN THE RABBIT : FACTORS INFLUENCING BIOAVAILABILITY AND CLEARANCE

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DECLARATION

In accordance with the regulations of the University of Edinburgh I declare that this thesis was composed by me and the work reported is my own except for those portions specified in the acknowledgements below.

Lynn M. Manson

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DEDICATION

I would like to thank Mum and Dad, for always being there.

HEPARIN PHARMACOKINETICS AND PHARMACODYNAMICS IN THE RABBIT: FACTORS INFLUENCING BIOAVAILABILITY AND CLEARANCE

Dr Lynn M. Manson

ABSTRACT

Unfractionated heparin has been the preferred anticoagulant for the prophylaxis and treatment of a wide range of conditions for over 50 years. Despite being used widely, its pharmacodynamic and pharmacokinetic characteristics remain poorly understood. Its clinical use is hindered by its poor bioavailability after subcutaneous injection, and as its half-life is also relatively short it must be administered therapeutically by continuous intravenous infusion. There is wide variation in dose response among patients and so the anticoagulant effect must be monitored closely by laboratory testing to optimise efficacy. Factors responsible for this variability have not been identified, but reversible heparin neutralisation by non-specific protein binding and increased heparin clearance in disease states have been implicated.

These disadvantages are overcome by the low molecular weight heparins. Their greater bioavailability at low doses and longer half-lives allow them to be administered to out-patients once or twice daily by subcutaneous injection and their more predictable anticoagulant response to weight-adjusted doses obviates the need for laboratory monitoring.

In order to improve our understanding of heparin bioavailability and clearance, and in an attempt to identify the factors responsible for variation in dose response, a reproducible in vivo model of heparin pharmacokinetics and pharmacodynamics was established in the rabbit. This has demonstrated that impaired heparin recovery is caused by increased non-specific protein binding. The extent of reversible protein binding is substantially less with low molecular weight than unfractionated heparin, and in contrast to unfractionated heparin which showed a significant reduction in recovery in experimental endotoxaemia, low molecular weight heparin does not bind to proteins that are released in response to endotoxin as its recovery was unaffected by acute endotoxaemia. The findings suggest that the binding proteins are likely to be proteins released from activated platelets, endothelial cells or leucocytes. There was no evidence that increased heparin clearance contributes to the reduced recovery observed in acute illness.

The novel in vivo application of low affinity heparin caused a significant improvement in the recovery and half-life of unfractionated heparin, and the clinical implications of obtaining the benefits of the low molecular weight heparins at a fraction of their cost by using the combination of low affinity and unfractionated heparins are great.

Although the results in rabbits may differ quantitatively from those in humans, they are likely to be qualitatively similar, and so it is probable that the variable anticoagulant response to heparin seen in patients is caused by variation in plasma protein binding rather than altered heparin clearance.

ABBREVIATIONS USED IN TEXT

AT III	Antithrombin III
AUC	Area under the curve
HAH	High affinity heparin
HC II	Heparin cofactor II
Heparin	Unfractionated and low molecular weight heparins
HPLC	High performance liquid chromatography
i.v.	Intravenous
LAH	Low affinity heparin
LMWH	Low molecular weight heparin
LPS	Lipopolysaccharide
NRS	Normal rabbit serum
PF4	Platelet factor 4
PPP	Platelet-poor plasma
RES	Reticuloendothelial system
SHPP	3-(4-hydroxyphenyl) propionic acid *N* - hydroxysuccinimide ester
UFH	Unfractionated heparin
vWF	von Willebrand factor

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CHAPTER 1

INTRODUCTION

INTRODUCTION

In Europe and North America each year, 2-4 percent of the population suffers a symptomatic thromboembolic event requiring anticoagulant therapy (van Beek, 1996). The standard management of such patients has for many years involved the in-patient administration of i.v. UFH for up to 10 days, followed by oral anticoagulant therapy for at least 3 months (Clark, 1995; Hirsh, 1991). UFH is an effective anticoagulant in the prevention and treatment of thromboembolic disease, and is also beneficial in the prevention of mural thrombosis after myocardial infarction, in the treatment of unstable angina, and in the prevention of coronary artery rethrombosis after thrombolysis. It is also used to prevent thrombosis in extracorporeal devices in cardiac surgery and haemodialyis, to treat selected cases of disseminated intravascular coagulation, and to prevent thrombosis in high-risk pregnancies. However, its clinical usefulness is limited by factors that determine its pharmacokinetic and pharmacodynamic characteristics. It has poor bioavailability at low doses, it must be administered parenterally, its half-life is dose-dependent, and the marked variability in dose-response makes it mandatory to monitor its anticoagulant effect closely by laboratoy testing.

There are two potential explanations for the marked variation in anticoagulant response to UFH. Firstly, non-specific binding of heparin to plasma proteins neutralises its anticoagulant activity and limits the amount of heparin that is available to bind to AT III, thereby decreasing its anticoagulant effect. Variations in levels of plasma proteins could result in variability in anticoagulant response. Secondly, the

underlying disease process may alter the function of components of the UFH clearance mechanisms such that UFH clearance may be increased and recovery reduced in the sick patient.

In recent years, low molecular weight derivatives of UFH have been prepared by a variety of techniques (Table 1), and have different pharmacokinetic and pharmacodynamic properties when compared with UFH. LMWHs have better bioavailability than UFH as they bind less to plasma proteins and they have a more predictable dose response than UFH. The effect of acute illness on the pharmacokinetics and pharmacodynamics of the LMWHs is unknown.

Table 1 : Comparison of LMWH preparations.

Preparation	Method of Preparation	Mean Molecular Weight	Anti-Xa : Anti-IIa ratio
Ardeparin	**Peroxidative depolymerisation**	**6000**	**1.9**
Dalteparin	**Nitrous acid depolymerisation**	**6000**	**2.7**
Enoxaparin	**Benzylation and alkaline depolymerisation**	**4200**	**3.8**
Nadroparin	**Nitrous acid depolymerisation**	**4500**	**3.6**
Reviparin	**Nitrous acid depolymerisation, chromatographic purification**	**4000**	**3.5**
Tinzaparin	**Heparinase digestion**	**4500**	**1.9**

Although UFH was first discovered over 80 years ago, its pharmacokinetics remain poorly understood. UFH is cleared predominantly by the saturable, or cellular

mechanism and although the elimination process was thought to follow first-order kinetics, the half-life of UFH increases as the dose administered increases. In contrast, LMWHs are eliminated from the circulation by the renal route. This clearance mechanism was thought to follow zero-order kinetics but surprisingly the half-life of the low molecular weight heparins is dose-independent, suggesting a first-order clearance mechansim. In addition, the half-life of LMWH is longer than that of a comparable dose of UFH because the clearance of LMWH by the renal route is slower than the clearance of an equivalent dose of UFH by the saturable route. The effect of acute illness on the mechanisms of clearance are unknown, and previous reports of their contributions to variability in anticoagulant response are inconsistent.

To investigate the factors responsible for the variable anticoagulant response to heparin in acute illness, an in vivo model of heparin pharmacokinetics and pharmacodynamics in the rabbit was established, and the results gained from its use are reported in this thesis. The in vivo clearance, recovery and metabolism of UFH and LMWH were compared, and the effect of acute inflammation on these parameters was assessed in endotoxin-treated animals. The components of the clearance mechanisms were identified, and attempts were made to modify factors that determine the bioavailability and clearance of UFH in order to overcome the characteristics that limit its clinical usefulness.

In accounting for the experimental results obtained it was thought necessary to consider the inherent differences in the biochemical and antithrombotic properties of UFH and LMWH. These are summarised in the following pages.

UNFRACTIONATED HEPARIN

Biochemical Structure

Commercial UFH is derived from mast cells of porcine intestinal mucosa and bovine lung and prepared as sodium or calcium salts. There is no evidence that the pharmacokinetic or biological properties of UFH are altered by its tissue source or the type of salt preparation. It is a strongly negatively-charged glycosaminoglycan consisting of chains of alternating uronic acid residues, which can be either D-glucuronic acid or L-iduronic acid, and D-glucosamine residues. The resulting polysaccharide chain is highly sulphated, with N-sulphate groups on the glucosamine residues and O-sulphate groups in various positions on both the uronic acid and glucosamine residues. Both the distribution of glucuronic acid and iduronic acid units and the sulphation pattern are variable (but not random) which results in a considerable structural heterogeneity. Commercial preparations of heparin are polydisperse, with molecular weights ranging from 3,000 to 30,000 Da (mean 12,000 to 16,000 Da), or 10 to 100 monosaccharide residues per chain.

Biological Activity

The anticoagulant activity of UFH is dependent upon its interaction with the naturally-occurring anticoagulant AT III, and its ability to accelerate greatly the rate by which AT III inactivates serine proteinases. The active centre serine of thrombin and other coagulation enzymes (including activated factors IX, X, XI and XII) is inhibited by an arginine reactive centre of the AT III molecule. UFH binds to lysine sites on AT III

and produces a conformational change at the arginine reactive centre whch converts AT III from a progressive, slow inhibitor to a very rapid inhibitor (Rosenberg and Bauer, 1994). Thrombin and activated factor X (factor Xa) are the serine proteinases that are most sensitive to accelerated inactivation by the heparin-AT III complex (Rosenberg and Bauer, 1994). The inactivation of thrombin by AT III in the presence of optimal concentrations of heparin is accelerated 2000- to 4000-fold, such that the half-life of free thrombin is reduced from 35 seconds to less than 1 second in the presence of heparin. An acceleration of comparable magnitude to that observed with thrombin has been shown for the reaction of AT III with factor Xa in the presence of heparin, reducing its half-life significantly from 90 seconds. The inactivation of other coagulation enzymes is accelerated to a much lesser extent, resulting in less of a reduction in the already relatively prolonged half-lives of factors IXa, XIa and XIIa of 10-25 minutes in the absence of heparin. Once it has induced the conformational change, UFH dissociates from the complex and can be reused (Rosenberg and Bauer, 1994).

Commercial UFH contains two fractions with greatly differing affinity for AT III. The high-affinity fraction, which constitutes about one third of the heparin chains, has high anticoagulant activity and accounts for about 90% of heparin's anticoagulant activity. The remaining low-affinity heparin (two-thirds of the chains) has low specific activity at less than 10% of that of the high-affinity fraction. The high-affinity fraction contains a unique pentasaccharide sequence containing a critically-positioned glucosamine residue (Choay et al, 1981; Rosenberg and Lam, 1979; Rosenberg and

Bauer, 1994; Lindahl et al, 1979; Casu et al, 1981) which is not found on the low-affinity heparin and which constitutes the specific AT III binding region of the high-affinity fraction. The heparin binding site of AT III has not yet been conclusively identified, but evidence exists that two arginine- and lysine-rich segments of the AT III molecule are involved, and these are the amino-terminal region up to about residue 50 (Blackburn et al, 1984; Gettins et al, 1992) and a region comprising approximately residues 100 to 145 (Peterson et al, 1987b; Smith et al, 1990).

Binding of AT III to heparin is a prerequisite for the ability of the polysaccharide to accelerate the inactivation of all target proteinases by the inhibitor, but the acceleration of the inactivation of certain proteinases also requires binding of the enzyme to heparin in a ternary complex. The inhibiton of thrombin requires that heparin binds to both AT III and the enzyme, but the inhibition of factor Xa requires that heparin binds only to AT III (Casu et al, 1981). Heparin molecules with fewer than 18 saccharide residues are unable to bind to thrombin and AT III simultaneously and cannot catalyse the inhibition of thrombin. In contrast, smaller heparin fragments are able to catalyse the inhibition of factor Xa by AT III, so long as they contain the high-affinity pentasaccharide binding site (Lindahl et al, 1984; Oosta et al, 1981; Nesheim, 1983).

Pharmacokinetics

The disappearance of heparin from plasma can be investigated either by using biological assays to monitor the anticoagulant activity of heparin in plasma

(pharmacodynamics) or by estimating the rate of disappearance of radiolabelled heparin (pharmacokinetics). The two methods differ in the fraction of heparin molecules that is measured. Only one-third of the molecules in commercial preparations of UFH bind AT III with high affinity (Andersson et al, 1976). The remaining two-thirds of fractions with low affinity to AT III have no anticoagulant effect in vitro and will not be detected by biological assays which are based upon the ability of heparin to accelerate the AT III-dependent inhibition of factor Xa. Therefore, the disappearance of the anticoagulant effect of heparin monitors the behaviour of approximately the one-third of UFH molecules with high affinity for AT III. The low-affinity fractions are still required for heparin to exert maximal antithrombotic effect, as fractions with only high affinity to AT III are less antithrombotic than UFH (Barrowcliffe et al, 1984). The radioactive isotope ^{125} iodine will bind to derivatised heparin (Greenwood et al, 1963) and this process is independent of heparin's affinity for AT III (Dawes and Pepper, 1979). Therefore, the disappearance of radiolabelled heparin from the vascular compartment assesses the rate of clearance of all heparin molecules regardless of their affinity for AT III, and is the 'net' product of the clearance of the different heparin moieties. The antithrombotic effect of the polydisperse UFH is monitored by the clearance of radiolabelled UFH from plasma, while the anticoagulant effect of its high-affinity fraction is assessed by biological assays, the majority of which are based upon AT III-dependent inhibition of factor Xa (Caranobe et al, 1986).

When supratherapeutic doses of UFH are injected intravenously in the rabbit, heparin

that has anticoagulant activity and is structurally intact is excreted in urine (Piper, 1947). Several hours later, the excreted heparin moieties have become desulphated and depolymerised (Dawes and Pepper, 1979). In contrast, when therapeutic doses of UFH are injected , only depolymerised and desulphated moieties are excreted (Piper, 1947; Dawes and Pepper, 1979). Desulphation probably precedes depolymerisation (Dawes and Pepper, 1979) and is likely to occur in the RES (Dawes and Pepper, 1979; Losito et al, 1977; Teien, 1977; Friedman and Arsenis, 1974; Oh et al, 1973; Bleiberg et al, 1983). Heparin has also been identified in vascular endothelial cells after parenteral injection (Hiebert and Jaques, 1976; Mahodoo et al, 1977) and endothelial cells in culture have been shown to have saturable binding sites for heparin (Glimelius et al, 1978; Barzu et al, 1985). Heparin is endocytosed by the endothelial cell (Barzu et al, 1985), and it is likely that the endothelium and/or the vessel wall contains heparin-metabolising activity (Barzu et al, 1987; Glimelius et al, 1978) and heparin-degrading activity (Barzu et al, 1987; Dawes and Pepper, 1992) but whether or not these processes occur in vivo has yet to be determined. After desulphation by the RES, the heparin metabolite is degraded to low molecular weight saccharides, possibly in the kidneys (Dawes and Pepper, 1979). A heparin-degrading endoglycosidase has been identified in rat spleen (Höök et al, 1977) but a similar enzyme has yet to be identified in human renal tissue. Together, these studies suggest that clearance of UFH from the intravascular space results from a widespread cellular mechanism of uptake followed by renal degradation of the released, desulphated product.

Initial work suggested that the half-life of UFH was dose-independent as clearance of the anticoagulant activity of therapeutic doses of i.v. heparin was exponential, consistent with a clearance mechanism that followed first-order kinetics (Olsson et al, 1963; Bjornsson et al, 1982). It was then observed that the half-life of heparin increased with the dose administered (Bjornsson and Levy, 1979; Olsson et al, 1963; Bjornsson et al, 1982; Estes, 1970), and that the pharmacodynamics of a large dose of UFH are best described by a concave-convex curve (de Swart et al, 1982). When this complex curve is resolved into its two components, it becomes apparent that UFH is cleared by a combination of saturable (cellular) and non-saturable (renal) mechanisms. The relative contributions of these two mechanisms is dependent upon the dose of UFH administered (Boneu et al, 1987a). At therapeutic doses, heparin is cleared predominantly by the cellular mechanism and the contribution of the renal mechansim to its clearance is small. At higher doses, when the cellular mechanism is saturated, heparin is cleared predominantly by the kidneys. Clearance studies in nephrectomised rabbits provide further evidence of the dose-dependence of heparin clearance mechanisms, as binephrectomy in the rabbit does not alter the clearance of low dose UFH but prolongs the clearance of a higher dose (Caranobe et al, 1985).

i) UFH -endothelial cell interactions

In vitro studies have shown that UFH binds to saturable sites on endothelial cells (Glimelius et al, 1978; Mahodoo et al, 1977), after which it is internalised and depolymerised (Mahodoo et al, 1977). The interaction of UFH with endothelial cells

in vivo results in the displacement of PF4 (Dawes et al, 1987). Endothelial binding sites for heparin have been characterised on both bovine (Vannucchi et al, 1988) and human (Barzu et al, 1985) cells, and endothelial uptake of heparin is likely to be a component of the saturable mechanism of clearance of UFH (de Swart et al, 1982). The cell receptors are not specific for heparin and also interact with other anionic polyelectrolytes, the affinity increasing with both charge and molecular weight (van Rijn et al, 1987). The bound UFH is highly antithrombotic but a large proportion is slowly endocytosed and metabolised (Vannucchi et al, 1988; Barzu et al, 1985; Dawes and Pepper, 1992). It is incorporated into the subendothelial matrix where it is catabolised to low molecular weight oligosaccharides devoid of anticoagulant activity. Although the small fragments may retain their AT III binding capacity (Barzu et al, 1987), they have no anticoagulant activity as the glycosaminoglycan undergoes complete desulphation which destroys its biological activity, followed by depolymerisation of the carbohydrate chain (Dawes and Pepper, 1992). In addition, heparin interacts with growth factors and affects coagulation, fibrinolysis and cell growth through endothelial-mediated mechanisms (D'Amore, 1990; Minter et al, 1992), many of which are independent of its anticoagulant activity (Sudhalter et al, 1989).

ii) UFH - RES interactions

Peripheral blood monocytes migrate from the marrow to tissues, spending only a few

hours in the blood. They continue to proliferate in the tissues where, as macrophages, their lifespan is measured in months or years. Macrophages have a prediliction for the liver and spleen. Some are fixed to endothelial structures lining the channels (sinusoids) of these organs and this fixed phagocytic capacity is called the RES. The cells of the RES play an important role in the metabolism of UFH (Fabian et al, !978). After i.v. injection, large amounts of radiolabelled UFH are taken up by the liver but the greatest concentration of radioactivity is found in the spleen (Dawes and Pepper, 1979). The plasma half-life of UFH in patients with cirrhosis is significantly prolonged compared with that of normal individuals and AT III activity is lower in normal individuals compared to that of cirrhotics, and so it is likely that UFH complexed to AT III is removed from the circulation by the liver (Teien, 1977). Once bound to specific receptors on the macrophage cell membrane, (Bleiberg et al, 1983), UFH is phagocytosed and fully desulphated before being returned to the circulation (Friedman and Arsenis, 1974; Dawes and Pepper, 1979, Wells and Dawes 1995). A heparin N-desulphamidase has been identified in various mammalian tissues (Dietrich, 1970) including rat spleen (Friedman and Arsenis, 1974) but O-desulphating activity has not yet been described. N- and O-desulphation results in loss of protein binding capacity and biological activity (Dawes and Pepper, 1979). Hepatectomy in the rat decreases the amount of desulphated heparin present in the circulation compared with normal rats (Wells and Dawes 1995). However, even in the absence of a functioning liver, heparin desulphation does occur and the vascular endothelium has been proposed as a third site of heparin metabolism (Wells and Dawes, 1995; Dawes and Pepper, 1992).

As the molecular weight of excreted heparin is much less than that of circulating oligosaccharides, UFH must undergo degradation prior to excretion (Dawes and Pepper, 1979). A heparin-degrading endoglycosidase has been isolated from rat spleen (Höok et al, 1977) but the site of degradation in man remains unknown. Significant amounts of radiolabelled UFH are associated with the kidney after i.v. adminstration (Dawes and Pepper, 1979) and it is possible that the kidney is the site of degradation. When UFH is administered in doses which saturate the cellular clearance mechanism, significant amounts are cleared by the non-saturable route and the intact UFH which is excreted is not desulphated. It therefore appears that UFH must undergo desulphation by the cells of the RES before degradation can occur (Dawes and Pepper, 1979).

Factors affecting bioavailability

UFH binding and neutralising proteins

In addition to its anticoagulant properties, heparin binds to cells (Barzu et al, 1986; Blieberg et al, 1983; Sobel and Adelman, 1988), influences cell growth (Clowes and Karnowsky, 1977) and binds to a large number of proteins (Lane, 1989) as a result of its strong anionic charge. Although the effect of the specific binding of heparin to AT III is well-documented (Rosenberg and Bauer, 1994), the physiological significance of many non-specific heparin-protein interactions is poorly understood. UFH binds to endogenous plasma proteins (such as histidine-rich glycoprotein, polymeric vitronectin, and fibronectin), to PF4 (released from activated platelets), and to high-

molecular weight multimers of vWF (released from platelets and endothelial cells) (Sobel et al, 1991; de Romeuf and Mazurier, 1993). Binding of UFH to plasma proteins reduces its anticoagulant activity by direct neutralisation and also by reducing the amount available to bind to AT III (Young et al, 1992). Most in vitro studies of protein-UFH interactions have used purified sytems and it is unknown if the in vitro findings are consistent with the protein-UFH interactions which occur in the in vivo plasma environment. There is evidence to suggest that in a whole plasma environment incorporating immobilised heparin, histidine-rich glycoprotein is the only plasma protein likely to neutralise a significant proportion of the anticoagulant activity of a therapeutic dose of UFH by direct competition with AT III (Dawes, 1993). Moreover, the contributions of individual protein-UFH interactions to the phenomenon of heparin resistance (when vast amounts of heparin are required to achieve a therapeutic anticogulant effect) are largely unknown (Andersen et al, 1981). Increased non-specific protein binding to UFH has been described in patients compared with healthy individuals (Young et al, 1992, 1993b) and it has been suggested that this difference is the result of increased levels of heparin binding proteins, some of which are likely to be acute phase reactants. Although the levels of many plasma glycoproteins increase during the acute phase response, increased synthesis of hepatic messenger RNA is not maximal until 8 hours after the initiation of the acute phase response, and increased plasma levels of acute phase proteins of hepatic origin occur several hours thereafter (Koj, 1974). However, the tendency for increased heparin requirements in heparin resistant patients is present only a few hours

after the onset of illness and initiation of the disease process (Young et al, 1992). Therefore, any plasma glycoprotein implicated in heparin resistance must be present in increased amounts in plasma within a few hours after the onset of the disease process. This significantly reduces the likelihood of proteins whose raised plasma levels are determined by increased de novo synthesis (such as traditional acute phase reactants of hepatic origin) playing a major role in heparin resistance.

LOW MOLECULAR WEIGHT HEPARINS

Biochemical Structure

In recent years, low molecular weight derivatives of commercial UFH have been prepared that have a mean molecular weight of approximately one-third that of UFH. Like UFH, LMWHs are glycosaminoglycans comprising chains of alternating residues of D-glucosamine and uronic acid, either L-iduronic or D-glucuronic acid (Rosenberg and Bauer, 1994). They are fragments of UFH manufactured by controlled enzymatic or chemical depolymerisation processes that produce chains with a mean molecular weight of about 5,000 Da and varying anti-Xa : IIa ratios. (Table 1).

Biological Activity

The LMWHs exert their anticoagulant effect in a manner identical to that of UFH. They bind to AT III through a unique pentasaccharide binding sequence which induces a conformational change in AT III and results in acceleration of the inhibition of serine proteases by the naturally-occurring anticoagulant. However, only 15 to

25% of LMWH chains contain the randomly distributed unique pentasaccharide binding sequence, compared with approximately one third of UFH chains. The principal difference between UFH and the LMWHs is in their relative inhibitory activity against factor Xa and thrombin (Harenberg, 1990). Less than 50 percent of LMWH chains are long enough to form the ternary AT III-thrombin-LMWH complex necessary to thrombin activity (Jordan et al, 1980). Therefore, unlike UFH which has equivalent activity against factor Xa and thrombin, LMWHs have greater activity against factor Xa compared with thrombin (Table 1).

Pharmacokinetics

The longer half-life, dose-independent clearance and better bioavailability of the LMWHs give them a more predictable anticoagulant response than UFH (Handeland et al, 1990). The plasma half-lives of LMWHs are two to four times as long as that of UFH. This difference results from the two glycosaminoglycans being cleared from plasma by different mechanisms. The LMWHs are cleared principally by the renal route because they have much lower affinity for endothelial cells (Barzu et al, 1984, 1987) and the macrophages of the RES (Bleiberg et al, 1983). The ability of UFH and LMWHs to bind to endothelial cells and macrophages may be dependent upon molecular weight and charge (van Rijn et al, 1987) , but other criteria, such as cellular affinity for AT III and characteristics of molecular fractions from other heparins, need to be examined before structural dependency can be confirmed.

Factors affecting bioavailability

Recovery of LMWH is greater than that of a comparable dose of UFH, and the bioavailability of LMWH after subcutaneous and i.v. administration is similar. When a low dose of LMWH is given subcutaneously, approximately 100 percent of the anti-factor Xa activity administered is recovered, compared with 30 percent with UFH (Bara et al, 1985), and the bioavailability after a subcutaneous dose is approximately 90 percent of an equivalent dose administered intravenously (Bratt et al, 1986). In addition, as the anticoagulant response to a given dose of LMWH is highly correlated with body weight (Mätzsch et al, 1987) LMWH is effective when given in standard weight-adjusted doses (factor Xa U/kg) and laboratory monitoring is unnecessary.

LMWH binding and neutralising proteins

Binding of proteins to LMWH differs greatly compared with that of UFH. Normal plasma is known to contain components which neutralise the anti-factor Xa activity of UFH but not smaller molecular weight fractions of heparin (Andersson et al, 1979; Lane, 1989), and this is of pharmacodynamic significance. Binding to plasma proteins may result in reduced recovery of UFH at low concentrations, variation in the anticoagulant response to a fixed dose of UFH in patients with thromboembolic disease (Hirsh et al, 1976) and the laboratory phenomenon of heparin resistance (Cruickshank et al, 1991). LMWH has lower affinity for histidine-rich glycoprotein (Lane et al, 1986), fibronectin (Dawes and Pavuk, 1991) and PF4 (Lane et al, 1986) than UFH, which may explain its superior bioavailability at low doses and the more

predictable anticoagulant response to high doses of LMWH (Handeland et al, 1990). As LMWHs have much lower affinity for heparin binding proteins than UFH, their plasma recovery is practically independent of dose and plasma concentration. However, non-specific plasma protein binding and neutralisation of LMWH anticoagulant activity does occur, and vitronectin and histidine-rich glycoprotein have been identified as important modulators of the anticoagulant activity of LMWHs (Dawes, 1993).

AIMS OF THE THESIS

It is apparent from the work discussed in this introduction that the understanding of the processes controlling the anticoagulant response to heparin in patients is less than complete. The objective of the investigations described in this thesis was to gain further knowledge of the factors responsible for variability in heparin dose response and included :

1. establishment of an in vivo healthy animal model for the study of heparin pharmacokinetics and pharmacodynamics;
2. comparison of the pharmacokinetics, pharmacodynamics, tissue localisation and metabolism of UFH and the LMWH enoxaparin;
3. assessment of the effect of endotoxaemic shock on heparin bioavailability and clearance;
4. identification of the factors that determine the anticoagulant response to heparin;
5. modification of factors identified as influencing heparin recovery in an attempt to

improve UFH bioavailability.

It was hoped that the results obtained would improve our understanding of the factors that determine the anticoagulant response to UFH and LMWHs in vivo in order to facilitate optimal prophylactic and therapeutic anticoagulation in the clinical setting.

CHAPTER 2

PHARMACOKINETICS AND PHARMACODYNAMICS OF UNFRACTIONATED AND LOW MOLECULAR WEIGHT HEPARINS IN THE RABBIT

INTRODUCTION

The disappearance of UFH from the vascular compartment can be assessed either by biological assays or by measuring the rate of disappearance of radiolabelled UFH. These two methods assess different characteristics of the heterogeneous commmercial UFH preparations. Initial biological assays used to measure the anticoagulant effect of UFH were global coagulation assays such as the whole blood clotting time or activated partial thromboplastin time, but these have been replaced by factor-specific assays because endogenous substances in blood contribute to the anticoagulant effect as assessed by global clotting assays (Godal, 1974). The biological effect of UFH is generally measured ex vivo using an assay based on anti-factor Xa activity because providing the AT III pentasaccharide binding sequence is present, the ability of UFH to accelerate the AT III-dependent inactivation of factor Xa is independent of chain length whereas its capacity to accelerate thrombin inactivation is chain-length dependent. However, only one-third of the molecules in commercial preparations of UFH (Andersson et al, 1976) and 15 to 25 percent of molecules in LMWH preparations contain the unique high-affinity pentasaccharide binding sequence (Harenberg, 1990). Thus, the anticoagulant clearance curve of UFH reflects the clearance of the high-affinity fractions only, while the elimination curve of radiolabelled UFH is a "net" summation of the more complex clearance curves of the different high- and low-affinity heparin fractions (Caranobe et al, 1986). Fractions with low affinity for AT III still have clinical significance because although they are devoid of an anticoagulant effect in vitro, they still have antithrombotic activity as

high-affinity fractions to AT III have less antithombotic effect than UFH (Holmer et al, 1982; Thomas et al, 1982; Barrowcliffe et al, 1984; Mattsson et al, 1989). The mechanism by which LAH enhances the antithrombotic effect of HAH is as yet unknown. Thus, in order to optimise clinical relevance, models of heparin clearance must be used which are not based solely on anticoagulant activity in order to give an accurate impression of clearance of antithrombotic activity.

Early investigations with UFH using global clotting assays suggested that heparin was cleared by a first-order exponential process (Estes et al, 1969; Olsson et al, 1963; Perry et al, 1974) as the clotting assay declined in a log-linear fashion. However, later studies using radiolabelled UFH (Dawes and Pepper 1979) and anti-factor Xa assays (de Swart et al, 1982) demonstrate that the pharmacokinetics of heparin are far more complex, as a characteristic concave-convex elimination curve is found (Boneu et al, 1989). The elimination of heparin is preceded by a fast initial removal of anticoagulant activity suggestive of an equilibration phase resulting from mixing of the drug in plasma and from distribution to other compartments. The complex concave-convex elimination curve cannot be explained by a simple kinetic linear model but the nonlinearity can be explained by an exponential mechanism (which is saturable) and a linear elimination mechanism (which is non-saturable) operating in parallel. The contribution of each mechanism to the overall clearance of glycosaminoglycans is dose- and type-dependent. The half-life of UFH is dose-dependent, increasing as the dose administered increases. At lower doses, the saturable mechanism is the principal route of UFH elimination, and this mechansim follows first-order kinetics. At higher

doses, this elimination mechanism is saturated and UFH is increasingly cleared by the nonsaturable route which follows zero-order kinetics. When therapeutic doses of UFH are administered, the zero-order component of clearance appears to be of minimal clinical significance and a dominant first-order process can be assumed (Kandrotas et al, 1989). However, the dose-dependent half-life of UFH can be explained by the change that occurs in the dominant clearance mechanism (from the rapid saturable to the slower non-saturable route) if the dose needs to be increased to achieve a therapeutic effect. The components of the saturable mechanism are cellular, and in vitro evidence suggests that UFH probably binds to endothelial and RES cells from where it is internalised, desulphated and depolymerised (Höök et al, 1977; Barzu et al, 1987; Bjornsson et al, 1988; Vannucchi et al, 1988). In contrast, the elimination of LMWH appears to occur almost solely by a non-saturable mechanism (Boneu et al, 1987b) and cellular binding plays a minimal role in LMWH clearance (Palm and Mattsson, 1987a). The half-life of LMWH is approximately twice as long as that of UFH and is not dose-dependent (Boneu et al, 1987b; Bara and Samama, 1988). The primary route of LMWH elimination appears to be renal (Palm and Mattsson 1987a, 1987b; van Rijn et al, 1987) and high concentrations of LMWH are found in the urine. The factors controlling this elimination process have yet to be described but it is unlikely that elimination occurs by passive glomerular filtration, since negatively charged heparin molecules will be repelled by the negatively charged heparan sulphate molecules that are abundant in the glomerular basement membrane (Kanwar and Farquhar, 1979). Further investigations are required to determine the pathophysiology

of renal glycosaminoglycan clearance.

It has long been known that there is wide variation in the anticoagulant response to a standard dose of UFH both in healthy volunteers and in hospitalised medical and surgical patients (Basu et al, 1972; Simon et al, 1978) and the most extreme example of this variability is exemplified by the laboratory condition of heparin resistance, when vast amounts of UFH are required to achieve a therapeutic level of anticoagulation (Cruickshank et al, 1991). There are a number of possible explanations for the increased heparin requirements seen in a proportion of patients treated with UFH. Increased UFH clearance in pulmonary embolism compared with venous thromboembolism may be caused by the disease process (Hirsh et al, 1976), but although similar findings can be reproduced in experimental pulmonary embolism, the underlying mechanism remains unclear (Chiu et al, 1977), and there is no evidence that heparin clearance is increased in cases of venous thromboembolism with excessive heparin requirements. Further investigation of the endothelial and reticuloendothelial macrophage mechanisms of UFH clearance is required to determine the contribution of the clearance mechanism to heparin resistance. Increased plasma levels of procoagulants (Edson et al, 1967) and reduced concentrations of AT III (Hirsh, 1986) theoretically could result in increased heparin requirements in order to achieve a therapeutic level of anticoagulation, but such abnormalities have not been found widely in clinical practice. There is evidence, however, that reversible heparin neutralisation (due to heparin binding to other plasma proteins which compete with AT III for heparin) is a major determinant of the

anticoagulant response to a fixed dose of UFH (Young et al, 1992). It has been suggested that variability in dosage requirements results from differences in concentrations of heparin neutralising proteins among patients, and those with heparin resistance will have the highest levels of neutralising plasma proteins. The identity of the neutralising proteins remains to be determined. Traditional acute phase reactants could play a role but the tendency to higher heparin requirements is present in patients' plasma very soon after the onset of the disease process even before heparin is commenced (Young et al, 1992). This does not allow sufficient time for upregulation of acute phase protein synthesis and release (Koj, 1974). In comparison, the recovery of LMWH is much less affected by non-specific binding to plasma proteins (Young et al, 1993a; 1994) which accounts for their better bioavailability. As the anticoagulant response to a fixed dose of LMWH is more predictable, the need for their close laboratory monitoring should be redundant.

In order to improve our understanding of the factors that determine the anticoagulant response to heparin in the clinical environment, an in vivo model of heparin clearance and recovery was established in the rabbit. Clearance of radiolabelled heparin from plasma , recovery of its biological activity, tissue localisation of radiolabelled heparin, and quantification and characterisation of excreted heparin were assessed in the rabbit after i.v. bolus injections of various doses of UFH, and the findings were compared with those using the LMWH enoxaparin.

METHODS

Animal studies

One of 3 doses of UFH (20, 50 or 100 anti-factor Xa U/kg) was administered by bolus i.v. injection to New Zealand White specific pathogen-free rabbits. Tracer amounts of radiolabelled UFH were administered intravenously at the same time. Serial blood samples were taken at various times over the next 60 minutes, when the animal was killed. A post mortem examination was performed, the bladder urinary volume was recorded, and representative samples of the spleen, liver, kidneys and urine were removed. The same experiments were performed with the LMWH enoxaparin.

Experiments were performed to assess the effect of LAH in vivo. Excess LAH (20-fold molar excess : UFH) was administered by bolus i.v. injection either 10 minutes prior to UFH (50 anti-factor Xa U/kg) or 15 minutes after it. Tracer amounts of radiolabelled UFH were administered intravenously with the UFH. Eight animals in total received LAH and UFH; each combination of LAH and UFH was administered to 4 animals. Serial blood samples were taken at various times in the 60 minutes following UFH administration, and PPP prepared. As a control, 2 rabbits were given only LAH and the small amount of anti-factor Xa activity measured in the plasma of these rabbits was subtracted from the anti-factor Xa activity measured in the plasma of rabbits that received UFH and LAH.

Clearance of ^{125}I - labelled heparin

The disappearance of radiolabelled heparin from plasma was plotted as a function of time and the elimination half-life ($t_{1/2}$) was determined by linear regression analysis after logarithmic transformation. The slope of the regression line which represents the elimination phase is designated -β and $t_{1/2}$ is 0.693/β (Gillies et al, 1986).

Heparin recovery based on anticoagulant activity

The anti-factor Xa activity of heparin in each plasma sample was determined by chromogenic substrate-based assays. To assess the amount of heparin that was bound non-specifically to plasma proteins (reversible heparin binding) ex vivo, the anti-factor Xa activity was measured before and after the addition of LAH.

Recovery of heparin in the first 20 minutes after heparin injection was calculated by plotting the plasma heparin concentration as a function of time and measuring the AUC using the trapezoidal rule method (Gillies at al, 1986). Analysis was restricted to the first 20 minutes after heparin administration because most of the anti-factor Xa activity of the lowest dose tested was cleared during this interval.

Quantification of organ radioactivity

The weight of each tissue sample was recorded and the amount of radioactivity in the tissue was determined in the gamma counter. The total amount of radioactivity per gram tissue was then calculated.

Quantification of urinary radioactivity

The bladder urinary radioactivity was calculated from the bladder urinary volume (obtained at post mortem examination) and the radioactivity in 100μL urine after radioactivity from unbound radiolabel had been subtracted.

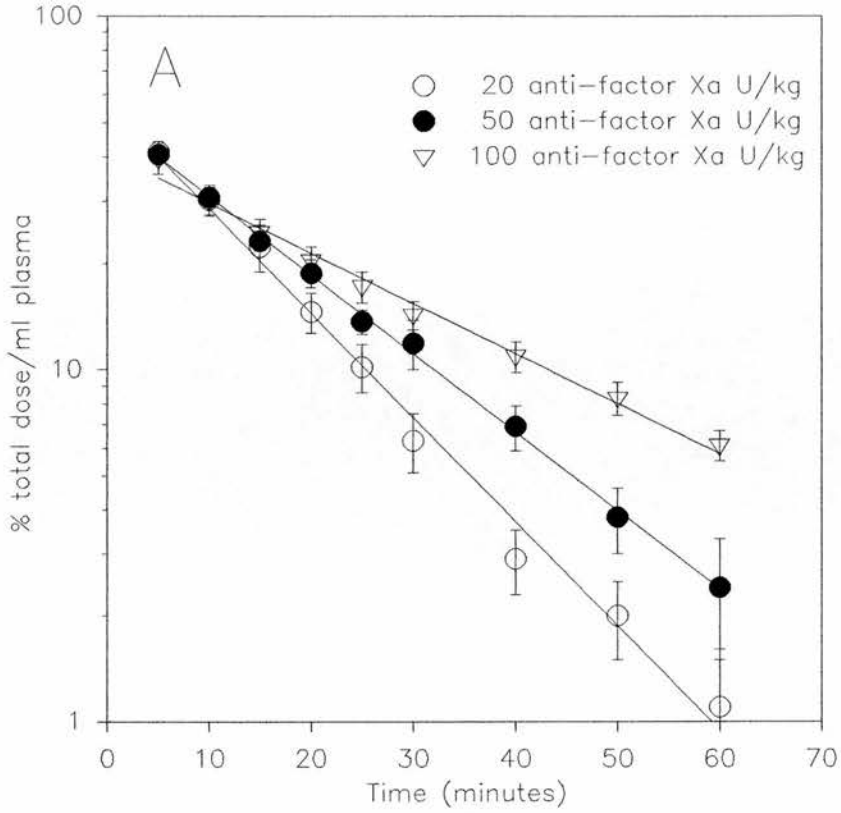
Characterisation of urinary heparin

Excreted urinary ^{125}I-heparin was separated using modified HPLC techniques and the radioactivity from urinary radiolabelled heparin was plotted as a function of time. The radioactivity-time profile was compared with those of non-metabolised radiolabelled UFH and LMWH in rabbit urine and the approximate molecular weights of the heparin fractions in the eluates containing peak radioactivity were determined.

RESULTS

Clearance of ^{125}I-labelled heparin

The elimination of radiolabelled heparin plotted as a function of time is shown in Figure 1. The duration of the distribution phase (5 minutes) and the rate of distribution were similar at all doses for both heparins. The plasma elimination half-lives of tracer amounts of ^{125}I-labelled heparin administered with various doses of excess unlabelled UFH and enoxaparin are shown in Table 2. The elimination of ^{125}I-UFH was dose-dependent and increased as the dose of unlabelled UFH increased. Thus, the $t_{1/2}$ after administration of 100 anti-factor Xa U/kg was 2.2 times longer than after 20 anti-factor Xa U/kg and 1.7 times longer than after 50 anti-factor Xa



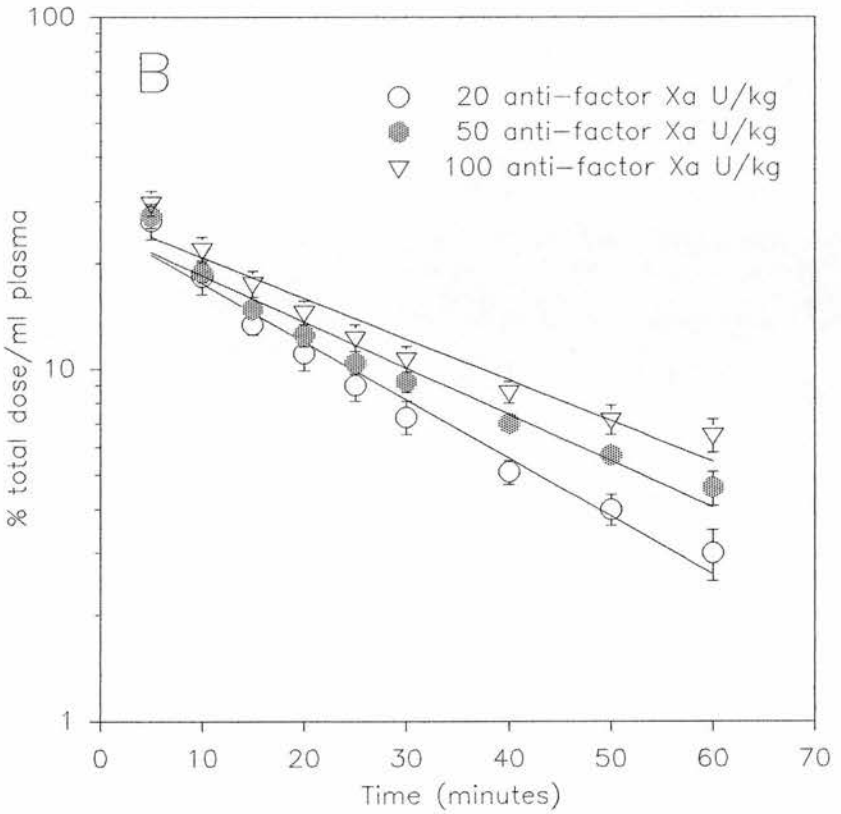


Figure 1A,B : Elimination half-lives of various doses of UFH (A) and enoxaparin (B) in control rabbits. Results presented are mean +/- SEM of 5 experiments.

U/kg. In contrast, the elimination of ^{125}I-enoxaparin showed some dose-dependency, although the half-lives increased only slightly with the higher doses of enoxaparin. Thus, the $t_{1/2}$ after administration of 100 anti-factor Xa U/kg was 1.4 times longer than after 20 anti-factor Xa U/kg and only 1.1 times longer than after 50 anti-factor Xa U/kg.

Table 2 : Plasma half-lives (minutes) of ^{125}I-labelled heparin administered with various doses of UFH and enoxaparin.

Dose (anti-factor Xa U/kg)	Plasma half-life (minutes)* UFH	LMWH	*p value*
20	**10.1 ± 1.0**	**18.4 ± 0.6**	**0.0004**
50	**13.6 ± 1.2**	**23.1 ± 1.0**	**0.0004**
100	**22.5 ± 0.7**	**26.1 ± 0.5**	**0.002**

Values are mean ± SEM of 5 experiments.

*** Plasma half-lives ($t_{1/2}$) were determined by linear regression analysis of the semi-logarithmic plot of plasma radioactivity v time.**

Recovery of heparin ex vivo

At each dose of heparin administered, there was a non-significant trend for greater non-specific protein binding of UFH than enoxaparin (Table 3).

Assessment of ex vivo and in vivo use of LAH

$AUC_{(0\text{-}20\ Unbound)}$ was significantly greater in animals that received LAH 10 minutes

Table 3 : Protein-bound UFH and enoxaparin in plasma of control animals in the first 20 minutes after adminstration of various doses of UFH and enoxaparin.

Dose (anti-factor Xa U/kg)	Bound UFH (%) *	Bound LMWH (%) †	*p value*
20	39.6 ± 4.5	27.3 ± 9.1	0.3
50	33.7 ± 2.7	15.3 ± 6.6	0.03
100	22.4 ± 3.7	13.9 ± 5.7	0.2

Values are mean ± SEM of 4 or 5 experiments.

*** Bound UFH is the amount of protein-bound UFH present in plasma in the first 20 minutes after administration of various doses of UFH. It is calculated from $AUC_{(0\text{-}20\ Total)}$ - $AUC_{(0\text{-}20\ Unbound)}$ and is expressed as a percentage of $AUC_{(0\text{-}20\ Total)}$.**

† Bound LMWH is the amount of protein-bound LMWH present in plasma in the first 20 minutes after administration of various doses of LMWH. It is calculated from $AUC_{(0\text{-}20\ Total)}$ - $AUC_{(0\text{-}20\ Unbound)}$ and is expressed as a percentage of $AUC_{(0\text{-}20\ Total)}$.

prior to 50 anti-factor Xa U UFH/kg compared with animals that received only 50 anti-factor Xa U UFH/kg ($p < 0.05$, Table 4A). In addition, $AUC_{(0\text{-}20\ Total)}$ in animals that received only 50 anti-factor Xa U UFH/kg was compared with $AUC_{(0\text{-}20\ Unbound)}$ in animals that received LAH 10 minutes prior to 50 anti-factor Xa U UFH/kg (Table 4B). There was no significant difference in UFH recovery between animals that received LAH ex vivo and ones that received LAH in vivo.

Table 4A : Effect of LAH in vivo on recovery of UFH from control animals.

	Unbound UFH (U ml^{-1}.min)*		
	- LAH †	+ LAH ‡	*p value*
Control	9.5 ± 0.6	15.5 ± 0.7	0.0001

Values are mean ± SEM of 4 (+LAH) or 5 (-LAH) experiments.

*** Unbound UFH is the amount of unbound UFH in plasma in the first 20 minutes after administration of 50 anti-factor Xa U UFH/kg. It is calculated from $AUC_{(0\text{-}20\ Unbound)}$.**
† 50 anti-factor Xa U UFH/kg were administered to rabbits in the absence of LAH.
‡ 50 anti-factor Xa U UFH/kg were administered to rabbits 10 minutes after LAH (20-fold molar excess : UFH) had been administered.

Table 4B : Comparison of ex vivo and in vivo quantification of reversible UFH neutralisation.

	LAH ex Vivo Total UFH (U ml^{-1}.min) *	LAH in Vivo Unbound UFH (U ml^{-1}.min) †	*p value*
Control	14.2 ± 0.6	15.5 ± 0.7	> 0.1

Values are mean ± SEM of 4 or 5 experiments.

*** Total UFH is the total amount of UFH in plasma in the first 20 minutes after administration of 50 anti-factor Xa U UFH/kg and is calculated from $AUC_{(0\text{-}20\ Total)}$.**
† Unbound UFH is the amount of unbound UFH in plasma in the first 20 minutes after administration of 50 anti-factor Xa U UFH/kg to animals that received LAH (20-fold molar excess : UFH) 10 minutes prior to the UFH. It is calculated from $AUC_{(0\text{-}20\ Unbound)}$.

Clearance of radioactivity and anticoagulant activity after LAH in vivo

When LAH (20-fold molar excess : UFH) was administered 15 minutes after UFH (50 anti-factor Xa U/kg), the resulting increase in plasma UFH concentration was not acccompanied by an increase in the amount of radiolabelled UFH in plasma (Figure 2). After LAH administration, there was an almost 70% increase in anti-factor Xa activity (from 0.47 ± 0.03 U/ml immediately before LAH administration to 0.79 ± 0.13 U/ml 5 minutes after LAH administration; $p = 0.06$). In contrast, there was no significant increase in the amount of radiolabelled heparin recovered in the plasma (17.0 ± 1.6 % of the total radiolabelled dose injected was present immediately before LAH administration and 17.7 ± 1.4 % was present 5 minutes later; $p = 0.75$). In addition, the administration of LAH in vivo resulted in an increase in the half-life of radiolabelled UFH such that its half-life was significantly longer than in the absence of LAH (13.6 ± 1.2 v 38.9 ± 0.9 mins, $p<0.01$) and was even greater than a comparable dose of enoxaparin (38.9 ± 0.9 v 23.1 ± 1.0 mins, UFH+LAH v enoxaparin) (Figure 2).

Tissue localisation of radiolabelled heparin

Unfractionated heparin

On a per organ basis, the liver accumulated the highest levels of radioactivity at all doses of UFH administered, with (11.6 ± 1.7)% to (30.8 ± 7.2)% of the total radiolabelled dose injected accumulating in the liver (Figure 3A). Localisation of radioactivity in the liver and spleen was dose-dependent, with the amount of

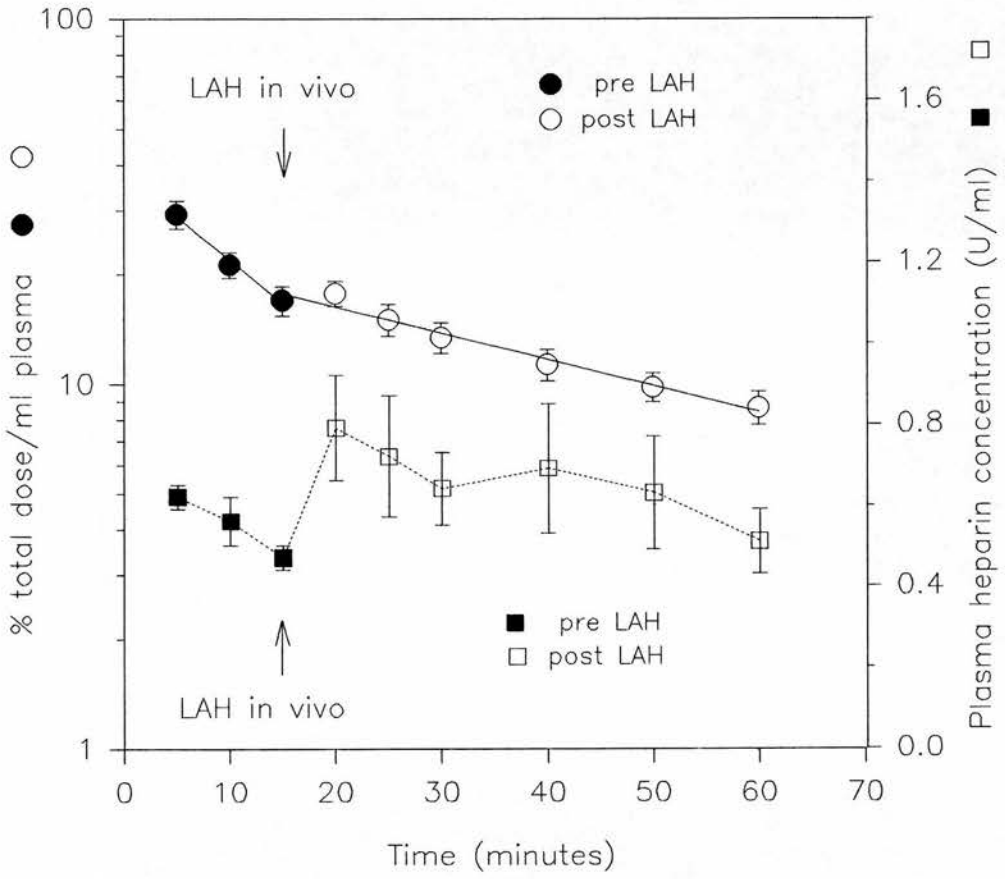


Figure 2 : Effect of LAH in vivo on UFH clearance and recovery. LAH was administered 15 minutes after a bolus i.v. injection of 50 anti–factor Xa U/kg of UFH to control animals. Results presented are mean +/– SEM of 4 experiments.

radioactivity detectable decreasing as the dose of carrier UFH increased. Localistion of radioactivity in the kidney was dose-independent, with no reduction in radioactivity detectable as the dose of carrier UFH increased (Figure 3A).

Low molecular weight heparin

On a per organ basis and on a per gram basis, the kidney accumulated the highest levels of radiolabelled LMWH at all doses of carrier LMWH administered (Figure3B). On a per organ basis, the kidney accumulated (8.1 ± 0.5)% of the total dose of radiolabelled enoxaparin injected, and thus approximately 16% of the total injected dose of radiolabelled enoxaparin localised in renal tissue. Localisation of radioactivity in the kidney was dose-independent, with no reduction in radioactivity detectable as the dose of carrier LMWH increased. Localisation in the liver and spleen was dramatically less than in the kidney, but in contrast to renal uptake, RES uptake was dose-independent (Figure 3B).

Quantification of urinary radiolabelled heparin

Small amounts of radiolabelled UFH were excreted. The amount of bladder urinary radioactivity was similar at all doses of UFH administered, and the mean amount of radiolabelled UFH excreted into the urine of control animals within 60 minutes of injection was (1.1 ± 0.2)% of the total dose injected. In contrast, at all doses studied approximately 12 times more enoxaparin than UFH was excreted; the mean amount of radiolabelled enoxaparin excreted was (13.8 ± 1.0)% of the total dose injected.

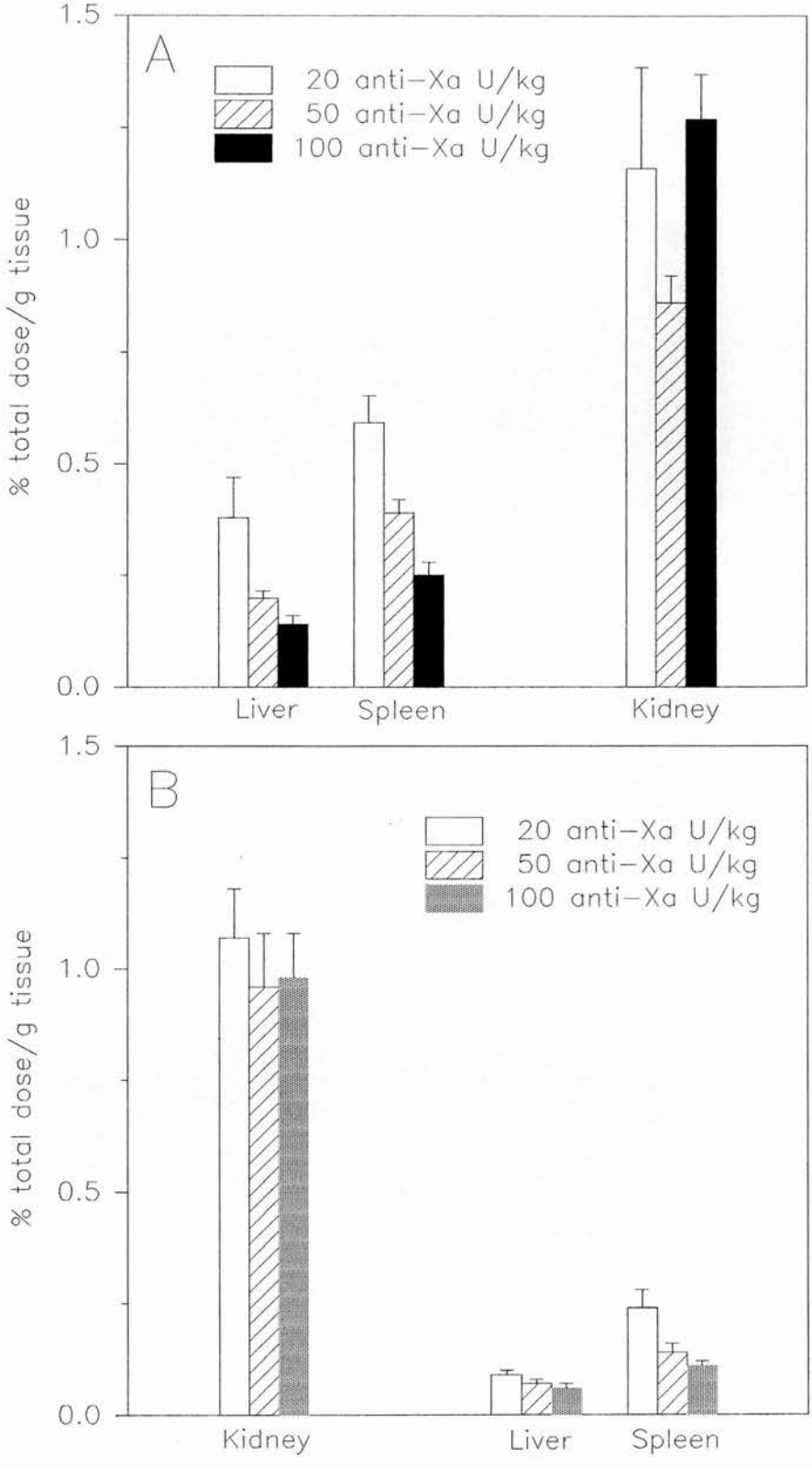


Figure 3A,B : Localisation of radioactivity in liver, spleen and kidney 60 minutes after a bolus i.v. injection of various doses of UFH (A) and enoxaparin (B) to control rabbits. Results presented are mean +/- SEM of 5 experiments.

Characterisation of urinary radiolabelled heparin

Chromatographic analysis of urine from control animals 60 minutes after a bolus i.v. injection of 50 anti-factor Xa U UFH/kg indicated that UFH had undergone degradation in vivo as the elution profile was similar to the non-metabolised iodinated low molecular weight compound (Figure 4A,B). In contrast, there was no evidence of in vivo degradation of enoxaparin as the elution profile was unchanged compared with the non-metabolised iodinated compound (Figure 4A,B).

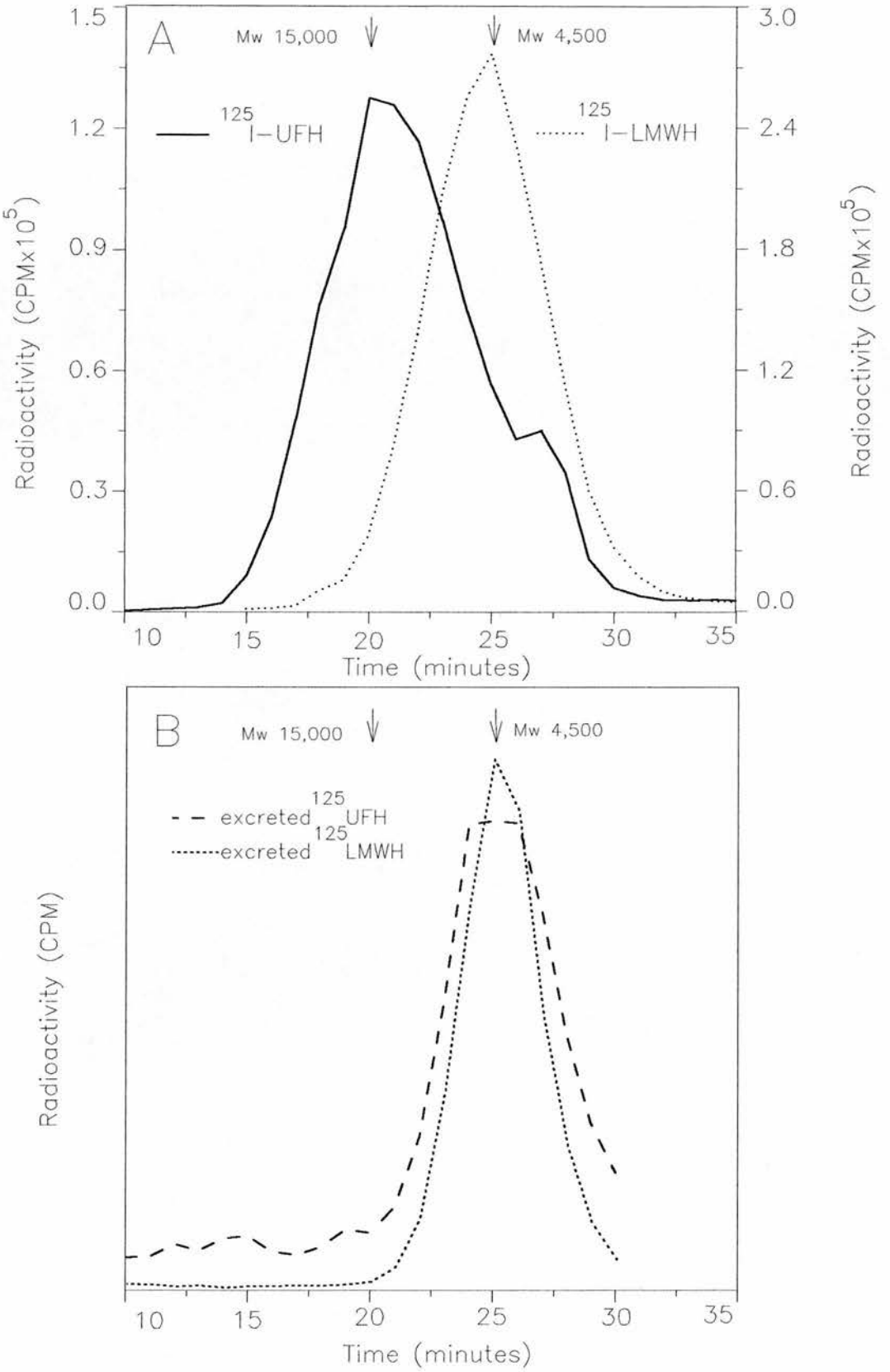


Figure 4 : HPLC profile of non-metabolised UFH —— and enoxaparin ····· in rabbit urine (A), and radiolabelled excreted UFH - - and enoxaparin ····· in urine from control rabbits (B).

DISCUSSION

The purpose of the study was to establish a reliable and reproducible in vivo model of heparin pharmacokinetics and pharmacodynamics in order to identify the factors responsible for the reduced heparin recovery observed in patients with high heparin requirements. The rabbit was chosen as the in vivo model because its coagulation system is similar to that of humans (Peyrou et al, 1997), and the size of animals facilitated blood sampling and organ extraction. Three doses of heparin were used in order to detect the effect of dosage on the various characteristics under study. The highest dose of UFH was chosen as it is equivalent to the loading dose administered by bolus i.v. injection to patients at the initiation of anticoagulant therapy, and is similar to the dose of UFH that is required to saturate the cellular clearance mechanism (Caranobe et al, 1985; Boneu et al, 1987a).

The disappearance of tracer amounts of radiolabelled heparin administered with various doses of excess cold (unlabelled) heparin was used to assess the clearance of heparin from the intravascular compartment. Previous work assessed the clearance of the biological activity of heparin from plasma (de Swart et al, 1982; Boneu et al, 1987a, 1987b) but this study has improved the clinical application of these observations by assessing the clearance of heparin that has antithrombotic activity, irrespective of whether or not it has an anticoagulant effect.

The clearance of radiolabelled UFH was dose-dependent, and the half-life increased as the dose of excess cold heparin was increased. In contrast, the clearance of radiolabelled enoxaparin was largely dose-independent, and the half-life increased only

slightly with the dose of unlabelled LMWH. At each dose studied, the half-life of enoxaparin was longer than that of UFH because the renal clearance of therapeutic doses of LMWH is slower than the cellular clearance of UFH (Caranobe et al, 1985). As anticipated, this difference was least marked with the highest heparin dose because at this dose a significant proportion of UFH has to be cleared through the slower renal route as the cellular mechanism is saturated (Boneu et al, 1987a, 1987b). These results agree with earlier findings and confirm the reproducibility and reliability of this animal model.

UFH binds to a number of plasma proteins in normal plasma (Lane, 1989) and only about 50% of its anticoagulant activity is recovered when it is added in vitro to normal plasma (Young et al, 1993a). The recovery of UFH is further reduced when it is added in vitro to patients' plasma because of non-specific binding to plasma proteins (Young et al, 1993b, 1994). In contrast, LMWH exhibits less plasma protein binding and at least 80% of enoxaparin is recovered in vitro from normal plasma (Young et al, 1993a, 1994). This study allowed similar observations to be made in vivo, although the difference between the two heparins in vivo was less striking than in vitro. Fifteen to 30 percent of enoxaparin was reversibly bound in vivo to plasma proteins which was comparable with in vitro findings, but only 20 to 40 percent of UFH was inactivated by non-specific binding to plasma proteins in vivo. This inconsistency may arise from differences between the in vivo and in vitro binding kinetics of UFH and plasma proteins which do not occur with enoxaparin. However, the in vivo and in vitro findings are consistent as enoxaparin exhibits less non-specific

plasma protein binding than UFH. Further studies are required to determine if this explains why patients with thrombosis treated with LMWH exhibit a less variable dose-response than those treated with UFH (Handeland et al, 1990).

LAH has been used extensively as an in vitro and ex vivo tool to determine the extent of reversible heparin neutralisation by non-specific plasma protein binding (Young et al, 1992, 1993a, 1994). Previous observations have been extended by the present novel use of LAH in vivo. This study has shown that LAH in vivo improves UFH recovery by displacing UFH from binding sites, and the extent of reversible heparin neutralisation that occurs in vivo can be assessed reliably ex vivo/in vitro using LAH. When LAH was given after UFH, it produced a marked increase in anticoagulant activity that was not accompanied by an increase in the amount of radioactivity present in plasma. These findings suggest that at the dose of UFH studied, the increase in anticoagulant activity after in vivo LAH administration is the result of displacement of UFH from circulating heparin binding proteins and is not caused by displacement of UFH from cellular sites. There are two potential explanations for these findings, namely that when given in clinical doses (i.e. 50 anti-factor Xa U/kg), UFH does not exhibit reversible binding to endothelial cells or macrophages in the RES. Alternatively, UFH is rapidly internalised by the cells of the RES and is thus inaccessible to displacement by LAH. This latter explanation appears the more likely as macrophages have been shown to internalise and degrade both UFH and a LMWH derivative (Bleiberg et al, 1983; Stehle et al, 1992).

The in vivo administration of LAH after UFH not only caused an increase in plasma

anticoagulant activity but also resulted in a significant prolongation of the half-life of UFH such that the clearance of radiolabelled UFH in the presence of LAH was slower even than the clearance of a comparable dose of enoxaparin. This suggests that in the presence of LAH, UFH can no longer be cleared by the cellular route and must now be cleared predominantly by the slower renal route. Identification of the principal components of the cellular mechanism of heparin clearance, and the effect of LAH in vivo on the uptake of radiolabelled UFH by these components is required to determine if this is indeed the case.

This study confirmed the previous observation that the principal organ of accumulation of UFH-derived radioactivity is the liver (Dawes and Pepper, 1979). It is established that phagocytic macrophages are widely distributed in the liver and spleen, and many are fixed to endothelial structures lining the sinusoids of these organs. An important role of the fixed macrophage is to clear unwanted particulate matter from the circulation. The findings in this model agree with earlier in vitro (Bleiberg et al, 1983; Falcone, 1989) and in vivo (Stehle et al, 1992) studies that proposed a macrophage-mediated receptor-dependent mechanism of UFH uptake by the liver. The results support the proposal that the principal organs of the RES, the liver and spleen, play a major role in the removal of UFH from the blood and extend previous findings by demonstrating in vivo that the uptake of UFH by the RES is dose-dependent, or saturable, while the uptake of LMWH is also saturable but is minimal. In contrast, the main organ of localisation of radiolabelled enoxaparin is the kidney. Thus, 60 minutes after injection, about 16% of injected radiolabelled

enoxaparin is localised in renal tissue and about 15% has been excreted in the urine in a non-metabolised form. These results agree with those of other workers who reported that LMWH is eliminated mainly from plasma via renal excretion (Boneu et al, 1987b; Palm and Mattsson, 1987b; Stehle et al, 1992) and extend these observations by demonstrating in vivo that the mechanism of renal clearance is associated with uptake of LMWH by renal cells. This can probably best be explained by a model in which a substantial proportion of LMWH that passes into the glomerular filtrate is taken up by renal cells before being excreted into the urine. The identity of these renal cells is uncertain, but a subgroup of mesangial cells has been described which is situated in the glomerulus and has phagocytic properties (Anderson and More, 1982), and could be responsible for the renal uptake of LMWH.

Approximately 12 times more radioactivity was measured in urine from animals that received enoxaparin compared with UFH. This finding confirms those of a previous study that showed that tritium-labelled preparations of another commercial LMWH (Fragmin) are eliminated from plasma predominantly by renal excretion while elimination of UFH is predominantly by metabolic clearance through the liver (Palm and Mattsson, 1987b). These observations confirm the use of iodinated heparin preparations as being a reliable method for assessing heparin pharmacokinetics in the rabbit.

There is a lack of a reliable chemical method for the direct measurement of administered heparin in biological fluids. Metachromatic assays have been used (Levy and Jaques, 1978; Gundry et al, 1984) but they are only suitable for tests on purified

materials and are insufficiently sensitive for investigations at the lower dose regimens now used therapeutically (Johnson and Mulloy, 1976). A competitive binding assay has been described which measures concentrations of administered heparin in urine (Dawes et al, 1985). However, the urine must firstly be deionised by dialysis and the low molecular weight cut-off dialysis tubing required (Mw 3,500) will potentially remove products of exogenous heparin catabolism. The urine must also be diluted to prevent competitive binding by endogenous glycosaminoglycans, which raises the detection limit above the concentrations expected in the animal samples. None of these methods provides molecular weight analysis of excreted heparin fractions. In this study, the approximate molecular weight of the excreted heparin compounds was assessed using a novel adaptation of HPLC. Initial attempts at molecular weight determination using standard Δ absorbance - time chromatograms were unsuccessful due to the presence in normal rabbit urine of significant quantities of carbonate and triple phosphate crystals (calcium carbonate monohydrate and ammonium magnesium phosphate). This particulate material interfered with light absorbance and various methods of removal and denaturation were unsuccessful. An alternative wavelength at which ΔOD of heparin but not urinary metabolites could be measured was not found. The presence of radiolabelled heparin compounds in urine was then exploited by counting the gamma-radioactivity present in the eluates obtained after free radiolabel had been removed by ethanol precipitation. Urinary radioactivity was detectable 60 minutes after a therapeutic dose of UFH had been administered by bolus i.v. injection, and the UFH had undergone degradation in vivo as the elution profile was

similar to the non-metabolised iodinated low molecular weight derivative. These results agree with those of Dawes and Pepper (Dawes and Pepper, 1979) who found a similar pattern of urinary radioactivity shortly after a therapeutic bolus injection of UFH had been administered, and the excreted isotope-containing material was also of low molecular weight (< Mw 1,000). They additionally found a second, larger peak of urinary radioactivity 5-9 hours after the injection. It is likely that the first urinary excretion coincides with the initial phase of heparin clearance, and is quantitaively small as the predominant method of UFH clearance is by hepatic metabolism. The second peak may be explained by release of heparin from sites of internalisation, such as endothelial cells, but further experiments are required to confirm this and are outwith the scope of this study.

Previous observations were extended by analysing urine from animals that had received enoxaparin. Although urinary radioactivity was also detectable 60 minutes after a bolus i.v. injection of enoxaparin, in contrast to UFH there was no evidence from this study that LMWH had undergone in vivo degradation as the elution profile was unchanged compared with the starting compound. Greater amounts of radioactivity were detected, consistent with the renal route being the principal clearance pathway. The most likely explanations for this lack of enoxaparin metabolism are firstly that RES uptake is an essential first step in heparin catabolism, and once internalised, heparin is metabolised before being broken down into saccharides. It follows that enoxaparin is excreted intact as cellular uptake of LMWH is minimal. If RES uptake of heparin is fundamental to its catabolism, blockade of

cellular uptake will result in excretion of non-metabolised UFH. Further experiments will be performed to determine if this is the case. Secondly, degraded enoxaparin molecules may have been present in the urine but remained undetected because their molecular size was below the detection limit of the method.

In conclusion, the observations recorded in this chapter confirm that the rabbit model described provides a reliable method for assessing the pharmacokinetics and pharmacodynamics of heparin in vivo.

CHAPTER 3

EXPERIMENTAL ENDOTOXAEMIA IN THE RABBIT : ITS EFFECT ON HEPARIN PHARMACOKINETICS AND PHARMACODYNAMICS

INTRODUCTION

As has been explained in some detail in the Introduction to this thesis, heparin exerts its anticoagulant effect by accelerating the AT III-mediated inhibition of blood coagulation factors. To do this, heparin must bind to AT III through a unique pentasaccharide binding sequence with high affinity for AT III which is present in approximately one-third of molecules (Choay et al, 1983; Lindahl et al, 1979; Höök et al, 1976; Lam et al, 1976). Heparin also binds non-specifically and reversibly to other plasma proteins which results in loss of its anticoagulant activity (Lane, 1989; McKay and Laurell, 1980; Zammit et al, 1993), and reduction in the amount of heparin available to bind to AT III. The proportion of anticoagulantly-active heparin that binds non-specifically to plasma proteins can be measured by the in vitro use of chemically-modified UFH, or LAH. This substance is devoid of anti-factor Xa activity and when added to normal heparin-containing plasma it displaces anticoagulantly-active heparin which is bound non-specifically to plasma proteins (Young and Hirsh, 1990; Young et al, 1993a).

There is wide variation in the anticoagulant response to a standard dose of UFH in patients with thromboembolic disease. Previous studies have shown that reversible heparin binding to plasma proteins is a major determinant of the anticoagulant response in patients (Young et al, 1992), and it has been suggested that patients who require large amounts of UFH do so because they have increased levels of heparin-binding proteins which cause excessive heparin neutralisation (Young et al, 1992). In contrast, LMWHs exhibit less binding to plasma proteins (Young et al, 1994) which

may explain their less variable anticoagulant response to a fixed dose (Prandoni et al, 1992; Hull et al, 1992; Weitz, 1997).

Variability in anticoagulant response to heparin has been seen in a variety of patient groups, including those with deep venous thrombosis (Cruickshank et al, 1991), pulmonary embolus (Hirsh et al, 1976; Simon et al, 1978) and sepsis (Simon et al, 1978). An inflammatory process is common to all these groups irrespective of the aetiology of the underlying disease. Inflammation is a curative process that results from any form of tissue injury, be it infective, traumatic, immunological or adrenergic in origin. It is accompanied by a diverse collection of local and systemic physiological changes which together constitute the acute phase response (Morrison and Ulevitch, 1978; Billiau and Vandekerckhove, 1991). The systemic response is mediated by cytokines released from activated macrophages that accumulate at the site of injury. These hormone-like substances act on many distant tissues to induce fever, endocrine changes, alterations in immune function, leucocytosis and changes in the concentration of some plasma proteins, known collectively as the acute phase proteins. This family of approximately 30 plasma proteins is generally produced in increased amounts by the liver in inflammation. Increased plasma concentrations are preceded by corresponding increases in hepatic mRNA concentrations, and the rates of secretion of some proteins also increase (Koj, 1974; Castell et al, 1989). Some of the heparin binding proteins such as fibronectin (Mosesson and Amrani, 1980), vitronectin (Preissner and Müller-Berghaus, 1987) and HC II (Tollefsen et al, 1982) are also acute phase reactant proteins (Hagiwara et al, 1990; Seiffert et al, 1995; Toulon et al, 1991), and so increased levels of these proteins during the acute phase

response could contribute to variabilty in anticoagulant response and heparin resistance. Against this proposition is the finding that the extent of reversible heparin neutralisation by non-specific plasma protein binding is determined early on in the disease process (Young et al, 1992). The potential for increased non-specific plasma protein binding is present in patients' plasma within a few hours of the onset of symptoms which is long before the de novo hepatic synthesis and release of acute phase reactants could have occurred (Koj, 1974). Alternatively, others have suggested that heparin requirements are increased in clinical pulmonary embolus because the clearance mechanism is altered by the disease process such that the half-life is significantly shortened (Hirsh et al, 1976; Simon et al, 1978).

In order to determine the relative contributions of reversible protein binding and altered clearance to the variability in anticoagulant response and the phenomenon of heparin resistance, an acute phase response was induced in rabbits and its effect on plasma heparin recovery, clearance and metabolism was studied.

METHODS

Induction of the acute phase response

A well-characterised model of experimental endotoxaemia was employed to assess the influence of the acute phase response on heparin pharmacokinetics and pharmocodynamics in the rabbit (Mathison and Ulevitch, 1979; Mathison et al, 1988). Initial dose-finding experiments were performed to establish the dosage of LPS required to promptly induce clinical symptoms of advanced endotoxaemic shock (Chapter 8). Administration of 10μg LPS/kg by bolus i.v. injection produced this state

within 2 hours, and by 3 hours the animal was moribund. Severe symptoms were produced more quickly with larger doses of LPS but this was considered unethical and not pursued; smaller doses had not produced clinically advanced endotoxaemia within 3 hours of administration.

Male New Zealand White specific pathogen free rabbits received 10μg LPS/kg by bolus i.v. injection 2 hours before 1 of 3 doses of UFH (20, 50 or 100 anti-factor Xa U/kg) was administered by bolus i.v. injection. Radiolabelled UFH in tracer amounts was added to the excess unlabelled anticoagulant before administration. PPP was prepared from serial blood samples taken at various time points over the next 60 minutes, and the animal was then killed. A post mortem examination was performed, the appearance of the lungs and spleen recorded, the bladder urinary volume was noted and representative samples of the spleen, liver, kidneys and urine were removed. The same experiments were repeated with the LMWH enoxaparin. Control animals were treated in the same way, but LPS was omitted. It was decided not to administer a sham bolus i.v. injection of saline to these animals 2 hours prior to the heparin to minimise animal handling and distress.

Clearance of ^{125}I-labelled heparin

Plasma radioactivity was plotted as a function of time and the elimination half-life ($t_{1/2}$) was determined by linear regression analysis after logarithmic transformation. The slope of the regression line representing the elimination phase is $-\beta$ and $t_{1/2}$ is $0.693/\beta$ (Gillies et al, 1986).

Heparin recovery based on anticoagulant activity

The anti-factor Xa activity of heparin in each plasma sample was determined by a chromogenic substrate-based assay, from which the plasma heparin concentration was determined.

Analysis of heparin recovery was restricted to the first 20 minutes after anticoagulant administration because at the doses studied, most of the anti-factor Xa activity was cleared during this period. Plasma heparin concentrations were plotted as a function of time and the AUC was measured using the trapezoidal rule method (Gillies et al, 1986).

Quantification of organ radioactivity

The radioactivity per gram tissue was calculated from the radioactivity and weight of each tissue sample.

Quantification of urinary radioactivity

The bladder urinary radioactivity was calculated from the bladder urinary volume (obtained at post mortem examination) and the heparin-bound radioactivity in 100μL urine.

Characterisation of urinary radioactivity

Urinary radiolabelled heparin was separated using modified HPLC techniques and the urinary radioactivity was plotted as a function of time. The approximate molecular

weights of the heparin fractions in the eluates containing peak radioactivity were determined.

RESULTS

Clearance of ^{125}I-labelled heparin

The duration of the distribution phase (5 minutes) and the rate of distribution were similar at all doses for each heparin type, and were not altered in the presence of experimental edotoxaemia. There was no significant difference in the elimination $t_{1/2}$ of ^{125}I-UFH in LPS-treated rabbits compared with controls at each dose of excess unlabelled UFH (Table 5). Similarly, there was no overall significant difference in the elimination $t_{1/2}$ of ^{125}I-enoxaparin in endotoxaemic animals compared with controls. At the lowest dose administered, the half-lives in the two groups were significantly different, but ^{125}I-enoxaparin was cleared more slowly from the circulation of endotoxaemic animals compared with controls (Table 5).

Recovery of heparin ex vivo

The amount of UFH bound to plasma proteins was markedly higher in the LPS-treated rabbits than in controls at each dose of UFH (Table 6A). The percentage of UFH bound non-specifically to plasma proteins was significantly higher (approximately 1.5-fold, $p<0.05$) in the LPS-treated animals that received 50 and 100 anti-factor Xa U/kg when compared to controls, and was close to significance ($p=0.08$) in the group that received 20 anti-factor Xa U/kg.

Table 5 : Plasma half-lives (minutes) of ^{125}I-labelled heparin in control and endotoxaemic animals after various doses of UFH and enoxaparin.

Dose (anti-factor Xa U/kg)		Plasma half-life (minutes) * Control	LPS-treated **	*p value*
UFH	20	10.1 ± 1.0	11.9 ± 1.1	0.28
	50	13.6 ± 1.2	16.8 ± 0.9	0.07
	100	22.5 ± 0.7	23.6 ± 1.9	0.60
LMWH	20	18.4 ± 0.6	20.9 ± 0.7	0.04
	50	23.1 ± 1.0	24.0 ± 0.5	0.45
	100	26.2 ± 0.5	24.4 ± 0.9	0.11

Values are mean ± SEM of 4 or 5 experiments.

*** Plasma half-lives ($t_{1/2}$) were determined by linear regression analysis of the semi-logarithmic plot of plasma radioactivity versus time.**

**** LPS was administered 2 hours before the heparin.**

Table 6A : UFH recovery in the first twenty minutes after administration of various doses of UFH to control and LPS-treated animals.

		1	2	3	4
Dose (anti-Xa U/kg)		Recovery - LAH ($U\ ml^{-1}\ .min$)	Recovery + LAH ($U\ ml^{-1}\ .min$)	Amount plasma-protein bound ($U\ ml^{-1}\ .min$)	% bound to plasma proteins
Control	**20**	**5.0 ± 0.6**	**8.4 ± 1.0**	**3.4 ± 0.7**	**39.6 ± 4.5 ***
	50	**9.5 ± 0.6**	**14.2 ± 0.6**	**4.8 ± 0.4**	**33.7 ± 2.7****
	100	**32.9 ± 1.9**	**42.9 ± 3.4**	**10.0 ± 2.3**	**22.4 ± 3.7****
LPS-treated	**20**	**3.5 ± 0.7**	**8.1 ± 1.1**	**4.6 ± 0.7**	**56.8 ± 6.7 ***
	50	**10.0 ± 1.5**	**17.6 ± 2.7**	**7.6 ± 1.2**	**43.4 ± 1.8****
	100	**28.9 ± 2.1**	**47.5 ± 6.4**	**18.4 ± 4.7**	**37.2 ± 5.5****

*** $p = 0.08$ ** $p < 0.05$**

In contrast, the recovery of enoxaparin was not altered by experimental endotoxaemia (Table 6B). At each dose of enoxaparin administered, there was no difference in the percentage of enoxaparin bound non-specifically to plasma proteins in LPS-treated animals compared with controls ($p>0.2$, control versus LPS-treated, at each dose).

Tissue localisation of ^{125}I-labelled heparin

Localisation of radioactivity in organs was essentially unaltered by experimental endotoxaemia. The liver and kidney remained the principal organs of uptake of radiolabelled UFH and enoxaparin respectively, and the amount of radioactivity that accumulated in each organ in LPS-treated animals was unchanged when compared

Table 6B : Enoxaparin recovery in the first twenty minutes after administration of various doses of enoxaparin to control and LPS-treated animals.

		1	2	3	4
Dose (anti-Xa U/kg)		Recovery - LAH ($U\ ml^{-1}$. min)	Recovery + LAH ($U\ ml^{-1}$. min)	Amount plasma-protein bound ($U\ ml^{-1}$ .min)	% bound to plasma proteins
Control	20	4.4 ± 0.4	6.3 ± 0.9	2.0 ± 0.8	27.3 ± 9.1 †
	50	10.8 ± 1.2	12.9 ± 1.5	2.1 ± 0.9	15.3 ± 6.6 †
	100	26.6 ± 6.6	27.7 ± 5.5	3.0 ± 1.2	13.9 ± 5.7 †
LPS-treated	20	5.2 ± 0.4	6.7 ± 0.6	1.6 ± 0.5	23.8 ± 6.6 †
	50	10.0 ± 0.7	13.6 ± 1.1	3.7 ± 0.7	26.4 ± 3.4 †
	100	28.0 ± 3.2	30.2 ± 3.8	5.0 ± 2.0	15.1 ± 3.9 †

† $p > 0.2$

Tables 6A,B : values are mean ± SEM of 4 or 5 experiments.

The amount of heparin recovered was determined as anti-factor Xa activity and was calculated from the AUC for the first 20 min after administration of heparin (Column 1). Excess LAH was added ex vivo to displace heparin from plasma protein binding sites and the AUC again calculated (Column 2). The difference between Column 1 and Column 2 is the amount bound to plasma proteins (Column 3) and is used to determine the percentage of heparin bound to plasma proteins (Column 4).
LPS was administered 2 hours before the heparin.

with control animals (Figure 5). At the lowest dose of UFH, there was a significant increase in the amount of radioactivity in the spleen of LPS-treated animals compared with controls ($p<0.01$). However, there was no difference between the control and endotoxaemic groups at either of the other 2 doses ($p>0.1$, at each dose), and overall,

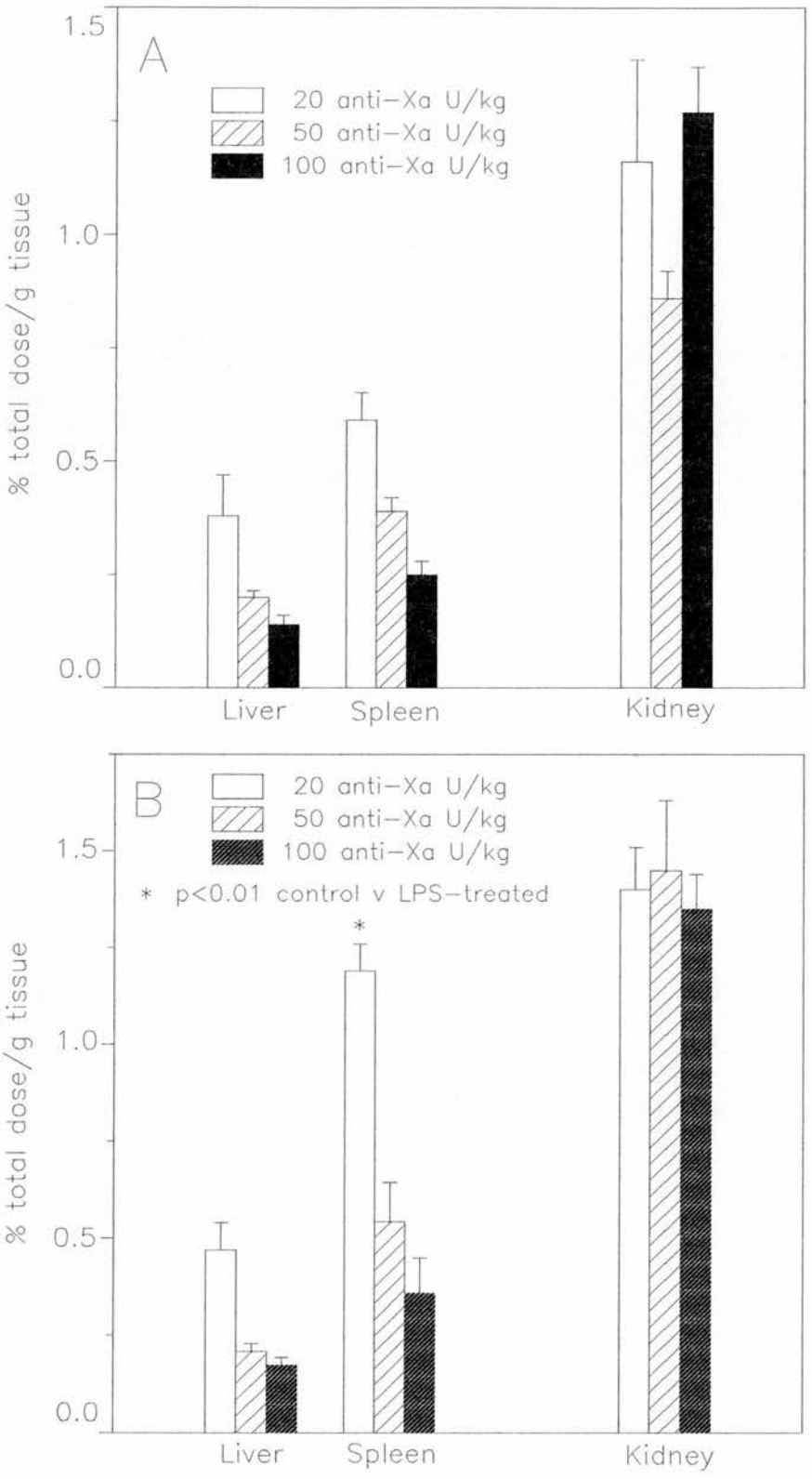


Figure 5A,B : Localisation of radioactivity in liver, spleen and kidney 60 minutes after a bolus i.v. injection of various doses of UFH to control (A) and LPS-treated (B) rabbits. LPS was given 2 hours prior to the UFH. Results presented are mean +/- SEM of 5 experiments.

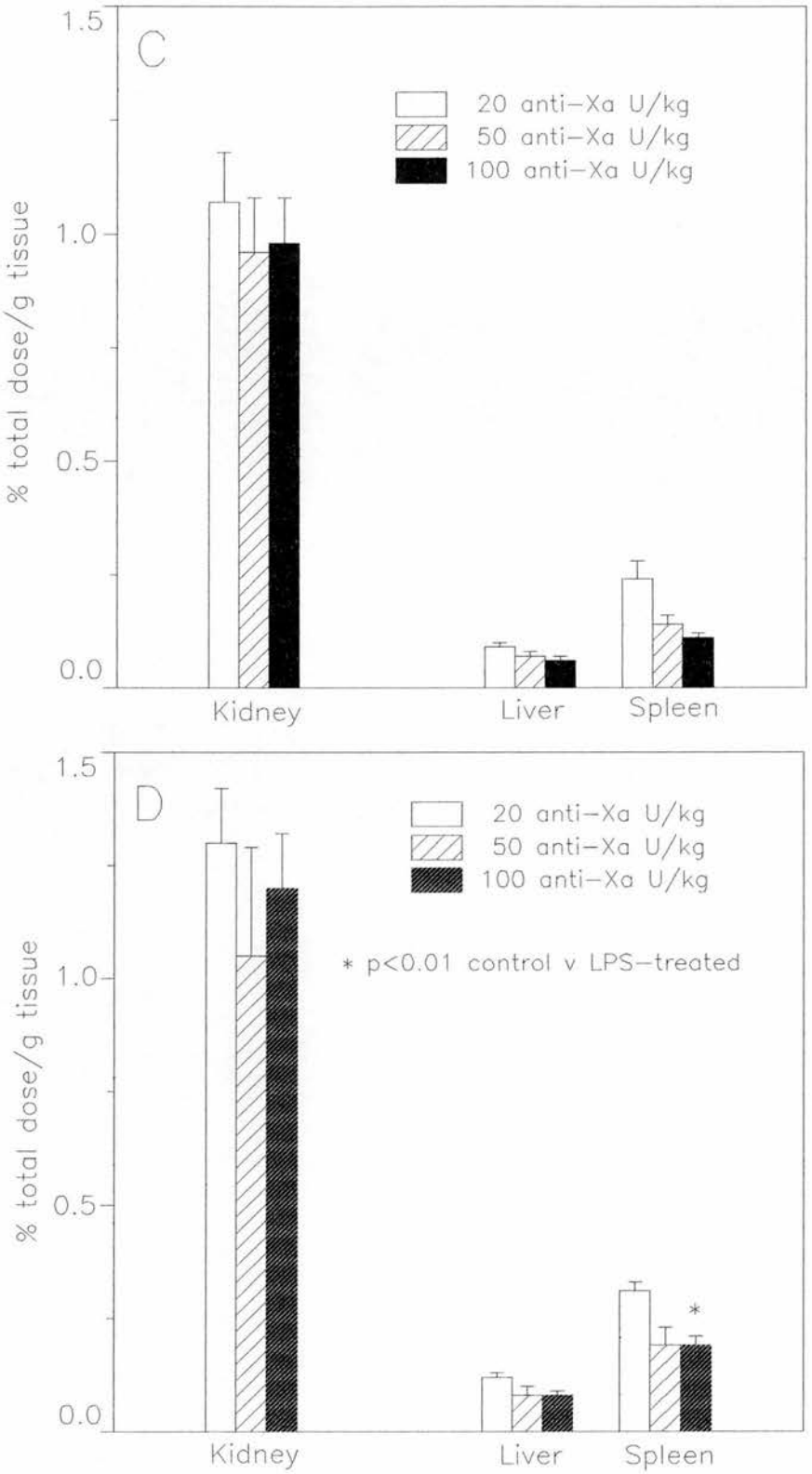


Figure 5C,D : Localisation of radioactivity in kidney, liver and spleen 60 minutes after a bolus i.v. injection of various doses of enoxaparin to control (C) and LPS-treated (D) rabbits. LPS was given 2 hours prior to the LMWH. Results presented are mean +/- SEM of 5 experiments.

there was no difference in the amount of radioactivity in the liver and kidney of endotoxaemic animals compared with controls ($p>0.1$ for each organ, at each dose, excepting kidney at 50 anti-Xa U/kg, when $p=0.02$). Similarly, at the highest dose of enoxaparin, there was a significant increase in splenic radioactivity in LPS-treated animals compared with controls ($p<0.01$). Once again, there was no difference between the control and endotoxaemic groups at either of the other 2 doses ($p>0.1$, at each dose), and endotoxaemia did not alter the amount of radioactivity in the liver and kidney when compared with control animals. The small increases in splenic radioactivity in endotoxaemic animals at the lowest dose of UFH and the highest dose of enoxaparin may therefore be regarded as being without biological significance.

Quantification and characterisation of urinary ^{125}I-labelled heparin

Experimental endotoxaemia did not produce a qualitative or quantitative change in excreted ^{125}I-labelled heparin. Despite advanced endotoxaemia, the role of the RES in heparin metabolism was preserved as in vivo metabolism of UFH continued (Figure 6) and, as in control animals, the amount of bladder urinary radioactivity in LPS-treated rabbits was dose-independent, and approximately 12 times more radiolabelled enoxaparin than UFH was excreted (Table 7).

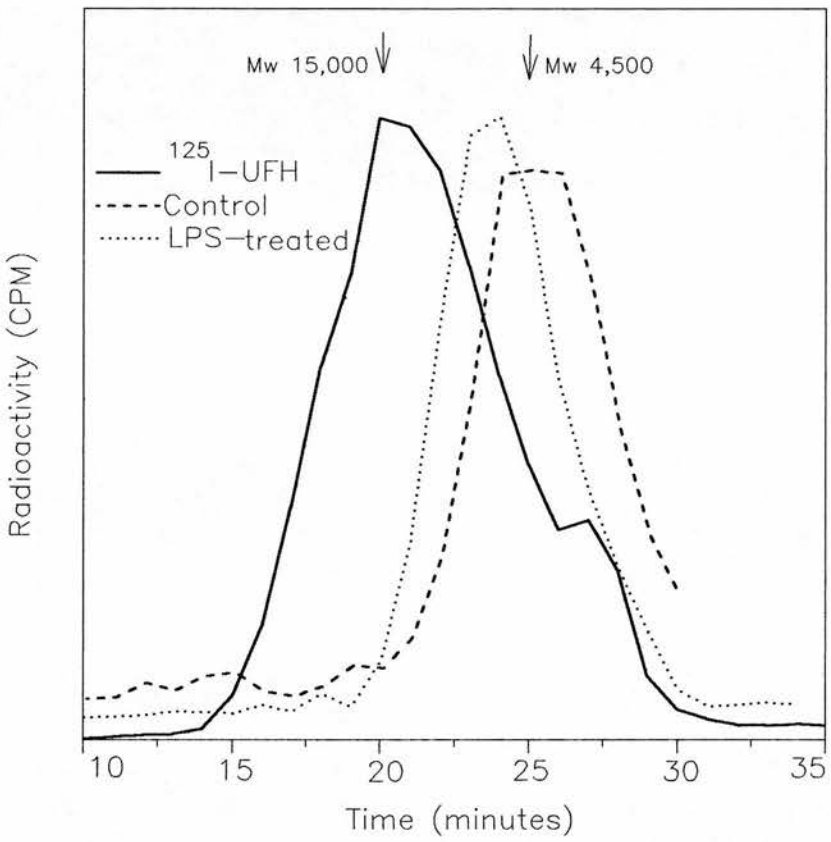


Figure 6A : HPLC profile of non–metabolised radiolabelled UFH in rabbit urine ——, and radiolabelled UFH in urine from control ---- and LPS–treated ····· rabbits. LPS was given 2 hours prior to the UFH.

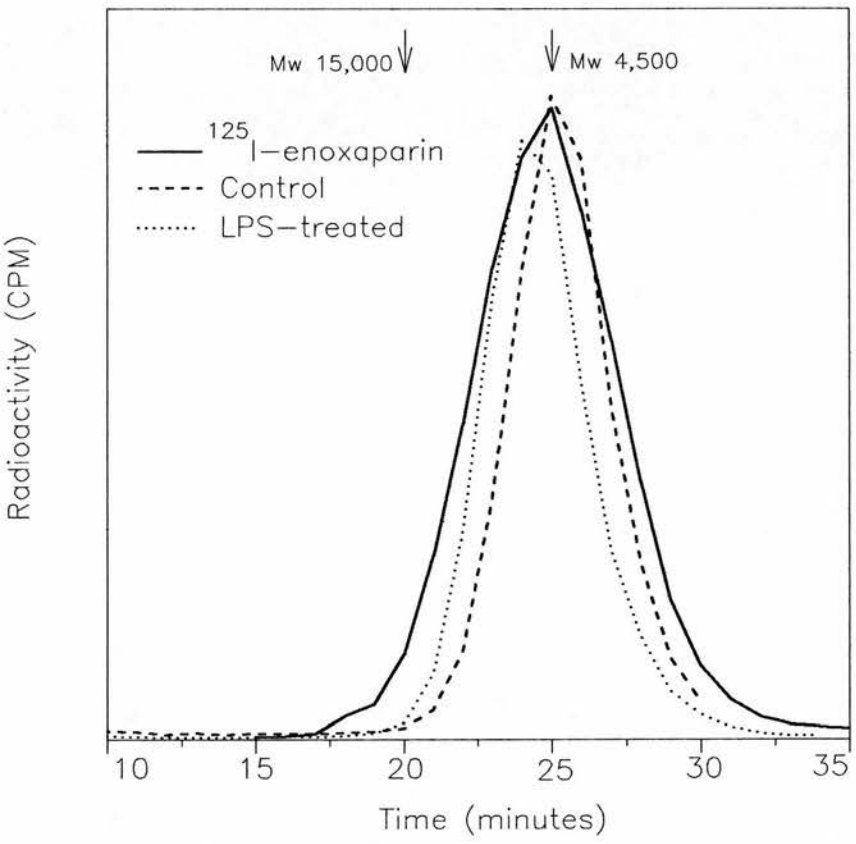


Figure 6B : HPLC profile of non–metabolised radiolabelled enoxaparin in rabbit urine ——, and radiolabelled enoxaparin in urine from control ---- and LPS–treated ····· rabbits. LPS was given 2 hours prior to the enoxaparin.

Table 7 : Excretion of I^{125}-labelled heparin in control and endotoxaemic rabbits 60 minutes after various doses of UFH and enoxaparin.

Dose (anti-factor Xa U/kg)		Bladder Urinary Radioactivity (% total dose) * Control	LPS-treated **	*p value*
UFH	**20**	**1.1 ± 0.1**	**0.6 ± 0.3**	**0.3**
	50	**0.8 ± 0.2**	**0.7 ± 0.2**	**0.7**
	100	**1.4 ± 0.6**	**0.9 ± 0.1**	**0.3**
	(mean)	**1.1 ± 0.2**	**0.8 ± 0.1**	**0.1**
Enoxaparin	**20**	**12.0 ± 1.8**	**15.2 ± 0.4**	**0.2**
	50	**14.7 ± 1.1**	**11.5 ± 2.7**	**0.3**
	100	**15.3 ± 2.2**	**11.7 ± 1.7**	**0.2**
	(mean)	**13.8 ± 1.0**	**11.8 ± 1.3**	**0.2**

Values are mean ± SEM of 3 to 5 experiments.

*** Bladder urinary radioactivity is the amount of radioactivity present in urine 60 minutes after a bolus i.v. injection of various doses of UFH or enoxaparin. It is expressed as a percentage of the total injected dose of I^{125}-labelled heparin corrected for free I^{125}.**

**** LPS was administered 2 hours prior to the heparin.**

DISCUSSION

The relative contributions of non-specific plasma protein binding and altered clearance to the variability in dose response to heparin and to the impaired anticoagulant response observed in some patients with acute venous thromboembolism are uncertain. In this study, the effect of acute inflammation on heparin clearance and reversible protein binding was examined. Inflammation is a curative process that is

common to all acute illnesses, and results from a variety of stimuli including infection, tissue injury, neoplastic growth and immunological disorders. An established experimental model (endotoxin administration) was employed to induce acute illness in rabbits. This model has been shown to increase the level of heparin binding proteins in rat plasma (Young et al, 1997) and to potentiate the development of venous thrombosis in rabbbits (Bernat et al, 1994). Endotoxin was administered intravenously in the form of LPS prepared from *Salmonella minnesota* Re595 bacteria. The dose administered was in excess of that which has been shown previously to induce maximal plasma levels of tumour necrosis factor (a major mediator of endotoxin-induced injury) within 100 minutes of i.v. administration (Mathison et al, 1988). A number of cell types including blood monocytes, tissue macrophages, neutrophils and platelets have also been shown to be activated by LPS (Morrison and Ulevitch, 1978). The results of this study show that the amount of UFH that is bound non-specifically to plasma proteins is markedly increased in the plasma of LPS-treated rabbits. This is similar to the effect of LPS on UFH recovery in the rat model (Young et al, 1997). Therefore, reduced heparin recovery in acute illness can be attributed to an increase in the plasma levels of heparin-binding proteins. In contrast, the recovery of enoxaparin was unaffected by acute endotoxaemia. Thus, although enoxaparin binds to a modest extent to plasma proteins in normal plasma, the results from this investigation indicate that unlike UFH it does not bind to the heparin binding proteins that are released into the circulation in response to LPS.

A dose of endotoxin was used that produced major multisystem perturbations within 2 hours of administration (Mathison et al, 1988). Since maximal hepatic synthesis of

acute phase protein mRNA does not occur until 8 hours after initiation of the acute phase response, and release of newly-synthesized acute phase proteins can therefore only take place many hours after the onset of inflammation (Koj, 1974; Castell et al, 1989; Hurlimann et al, 1966) it may be concluded that the model of acute inflammation used in this study predominantly assessed the extent of reversible heparin binding to proteins that are released from storage sites in endothelial and other vascular cells in response to endotoxin injury. It was felt essential to design a model that would assess this aspect of reversible heparin neutralisation because the tendency to increased heparin requirements occurs early in the disease process, before plasma levels of acute phase reactants have risen (Young et al, 1992). As cytokine-mediated release of PF4, lactoferrin and vWF from cellular storage sites occurs during the inflammatory response (Fiedel, 1988; Billiau and Vandekerckhove, 1991; Schorer et al, 1987) these heparin binding proteins (Rucinski et al, 1990; Maccarana and Lindahl, 1993; Wu et al, 1995; de Romeuf and Mazurier, 1993) are implicated in increased reversible heparin neutralisation in experimental endotoxaemia. A similar situation may occur in humans when patients with thrombotic disorders show a reduced anticoagulant response to heparin within a few hours of onset of their symptoms. Finally, the findings from this study provide an explanation for the observation that patients with thrombosis treated with LMWH exhibit a less variable dose response than those treated with UFH (Handeland et al, 1990).

These results demonstrate that reversible heparin binding is an important mechanism for reduced heparin recovery in acute illness, and that greater variability in anticoagulant response to UFH than LMWH can be expected. In contrast, there is no

evidence of increased heparin clearance in experimental endotoxaemia. Thus, the elimination half-life of heparin in endotoxaemic rabbits was not different from that in controls, even in the endotoxaemic animals that received UFH who had markedly reduced recovery. This finding supports the observation that acute venous thrombosis does not reduce heparin half-life (Hirsh et al, 1976) although it is at variance with the finding that heparin clearance was reduced in clinical and experimental pulmonary embolism (Hirsh et al, 1976; Chiu et al, 1977; Simon et al, 1978). The cause of this inconsistency is not known but it may be ascribed to differences in the nature of the thrombi found in venous thrombosis and pulmonary embolism. Further support for the lack of a role for altered clearance in the variation in heparin dose-response is provided by the absence of effect of experimental endotoxaemia on tissue localisation of radiolabelled heparin, and the amount and characteristics of excreted radiolabelled heparin, consistent with the absence of effect of endotoxaemia on half-life.

In summary, using an animal model of acute endotoxaemia, the phenomenon of impaired heparin recovery has been demonstrated to be caused by an increase in the concentration of heparin binding proteins. In contrast to UFH which showed a significant reduction in recovery in endotoxaemic animals, the recovery of the LMWH enoxaparin was not altered by endotoxaemia as it did not bind to the neutralising proteins that increase after endotoxin treatment. There was no evidence that increased heparin clearance contributes to the reduced heparin recovery observed in acute illness. Although the results in rabbits may be quantitatively different from those in humans, they are likely to be qualitatively similar. These findings suggest that the variable anticoagulant response to UFH seen in patients with venous thrombosis is

likely to be consequent upon alterations in plasma protein binding rather than heparin clearance, and explain why patients with thrombosis exhibit a less variable dose-response when treated with LMWH compared with UFH.

CHAPTER 4

NOVEL IN VIVO APPLICATION OF A LOW AFFINITY HEPARIN TO IMPROVE HEPARIN RECOVERY

INTRODUCTION

After almost two decades of research, LMWHs are now established as important antithrombotic compounds that offer considerable advantages over their parent preparation, the more traditional antithrombotic UFH. In the mid and late 1980s, it was reported that low molecular weight preparations had a longer plasma half-life and better bioavailablity at low doses than UFH (Frydman et al, 1988; Briant et al, 1989; Bratt et al, 1986; Mätzsch et al, 1987; Bara and Samama, 1988; Bradbrook et al, 1987), and a more predictable dose response (Handeland et al, 1990). These pharmacokinetic and pharmacodynamic advantages are now known to result from differences in plasma protein and endothelial cell binding that exist between the two heparin preparations.

In 1979 the observation was made that normal plasma will neutralise the anti-factor Xa activity of UFH but not of smaller molecular weight fractions of heparin (Andersson et al, 1979). This neutralising activity of plasma was found to be caused by plasma proteins which compete with AT III for heparin binding, and the non-specific binding was found to vary with the molecular size of the heparin. Many plasma and platelet proteins with non-specific heparin binding activity have been described, including PF4 (Lane et al, 1986; Holt and Niewiarowski, 1985), vWF (Sobel et al, 1991), histidine-rich glycoprotein (Lane et al, 1986; Lijnen et al, 1983; Peterson et al, 1987a) and lipoporoteins (Lane, 1989). It is likely that non-specific protein binding contributes to the reduced bioavailability of UFH at low concentrations, the variability in anticoagulant response to fixed doses in patients with

thromboembolism (Hirsh et al, 1976) and to the laboratory phenomenon of heparin resistance (Cruickshank et al, 1991; Young et al, 1992; Young et al, 1993a) In contrast, LMWHs have lower affinity than UFH for many neutralising proteins, including histidine-rich glycoprotein, PF4 (Lane et al, 1986) and vWF (Sobel et al, 1991). This relative lack of protein binding of the LMWHs gives them three important practical advantages over UFH, namely superior plasma recovery when administered in low doses, a relative lack of resistance to their anticoagulant effect, and a more predictable anticoagulant response to weight-adjusted doses.

The molecular size of UFH and its fragments also influences binding to cellular components. UFH binds to endothelial cells and is then internalised (Barzu et al, 1985), whereas the LMWHs do not bind to endothelial cells in culture (Barzu et al, 1984). The reduced binding of LMWHs to the endothelium contributes to their greater bioavailability compared with UFH. In addition, the LMWHs show a reduced tendency to bind to macrophages compared with UFH (Bleiberg et al, 1983) which results in a longer plasma half-life. Therapeutic doses of UFH are cleared predominantly by the saturable, or cellular, mechanism. The principal component of this mechanism is the macrophage-rich RES and the liver is the main organ of UFH uptake (Dawes and Pepper, 1979; Losito et al, 1977; Teien, 1977). In contrast, LMWHs bind minimally to macrophages (Stehle et al, 1992), and so are cleared predominantly by the renal route (Boneu et al, 1987b). This method of clearance is slower than that of UFH when both heparins are administered at their usual therapeutic doses (Boneu et al, 1987b; Caranobe et al, 1985), resulting in the longer

half-life of LMWH compared with UFH.

Thus, the disadvantages of non-specific plasma protein binding and RES uptake have been overcome by the LMWHs, produced commercially by controlled enzymatic and chemical depolymerisation of UFH. The processes required for commercial production are costly and the LMWHs are approximately 4 times more expensive to use clinically than UFH. It would therefore be beneficial if the disadvantages of UFH could be overcome in such a way that the benefits of the LMWHs could be bestowed on UFH at a fraction of the cost. Various agents have been used in vivo which block RES uptake (Bleiberg et al, 1983; Palm and Mattsson, 1987b; Stehle et al, 1992) but any resulting prolongation in duration of biological activity is offset by reduced intensity of activity consequent upon unopposed non-specific protein binding. Therefore, in an attempt to improve UFH recovery, the molecular weight dependence of both RES uptake and non-specific protein binding of UFH was exploited by the novel in vivo administration of a chemically-modified UFH which is essentially devoid of anticoagulant activity but identical in all other respects to the parent compound.

METHODS

Preparation of LAH

LAH was prepared from unfractionated porcine mucosal heparin by controlled periodate oxidation and borohydride reduction (Casu et al, 1986) with minor modificatons (Young and Hirsh, 1990). The LAH anti-factor Xa activity was less than 1.0 U/mg.

Animal Studies

Excess LAH (20-fold molar excess : heparin) was administered by bolus i.v. injection 10 minutes prior to heparin (50 anti-factor Xa U/kg) and some animals were pretreated with LPS. The following groups were studied :

1. ***LAH prior to heparin*** : excess LAH (20-fold molar excess : UFH) was administered to 8 rabbits 10 minutes before a bolus i.v. injection of UFH (50 anti-factor Xa U/kg). Tracer amounts of radiolabelled UFH were administered with the unlabelled anticoagulant. Blood samples were taken at various times over the next 60 minutes, and PPP was prepared. Four of the animals had received LPS (10μg/kg) intravenously 120 minutes prior to the UFH. After 60 minutes, the animals were killed. A post mortem examination was performed, the macroscopic appearance of the spleen and lungs was recorded, the bladder urinary volume was noted and representative samples of the spleen, liver, kidneys and urine were removed. The experiments were repeated using enoxaparin in place of UFH.

2. ***Anticoagulant activity of LAH in vivo*** : as a control, 2 rabbits received a bolus i.v. injection of LAH (20-fold molar excess : UFH, assuming 50 anti-factor Xa U UFH/kg had been administered) and 10 minutes later a further injection of physiological saline. Blood samples were taken at various times in the 70 minutes after LAH administration and PPP prepared. LPS had been administered to 1 of the animals 110 minutes prior to the LAH. After 70 minutes, the animals were killed. A post mortem examination was performed and the macroscopic appearance of the lungs and spleen was recorded. The same experiments were repeated using LAH at a dose of 20-fold

molar excess : LMWH, assuming 50 anti-factor Xa U LMWH/kg had been administered.

Clearance of ^{125}I-labelled heparin

The disappearance of radiolabelled heparin was plotted as a function of time and the elimination half-life ($t_{1/2}$) was determined by linear regression analysis after logarithmic transformation. The slope of the regression line which represents the elimination phase is designated -β and $t_{1/2}$ is 0.693/β (Gillies et al, 1986).

Heparin recovery based on anticoagulant activity

The anti-factor Xa activity of heparin in each plasma sample was determined by chromogenic substrate-based assays, and the plasma heparin concentration was determined from this value.

Recovery of heparin was calculated by plotting the plasma heparin concentration as a function of time and measuring the AUC using the trapezoid rule method (Gillies et al, 1986). Recovery was assessed both in the first 20 minutes and the full 60 minutes after heparin administration.

Quantification of organ radioactivity

The total amount of radioactivity per gram tissue was calculated from the radioactivity and weight of each tissue sample.

Quantification of urinary radioactivity

Bladder urinary radioactivity was calclulated from the bladder urinary volume (obtained at post mortem) and the radioactivity from ^{125}I-labelled heparin in 100μL urine.

Characterisation of urinary heparin

Excreted radiolabelled heparin was separated using modified HPLC techniques and the radioactivity from ^{125}I-labelled heparin in urine was plotted as a function of time. The approximate molecular weights of the heparin fractions in the eluates containing peak radioactivity were determined.

RESULTS

Tissue localisation of radiolabelled heparin

Administration of LAH had a similar effect in control and endotoxaemic animals, and so findings in control animals are considered representative. Localisation of radiolabelled heparin in the liver and spleen was reduced significantly by pre-administration of LAH ($p<0.02$ for each organ [-LAH v +LAH], Figure 7). However, in absolute terms, pre-administration of LAH had a more profound effect on localisation of ^{125} I-UFH than ^{125} I-enoxaparin. The absolute amount of radiolabelled UFH that localised in the liver and spleen was reduced markedly by pre-administration of LAH (Figure 7A). In contrast, pre-administration of LAH reduced the amount of radiolabelled enoxaparin that localised in the liver and spleen from slight to almost

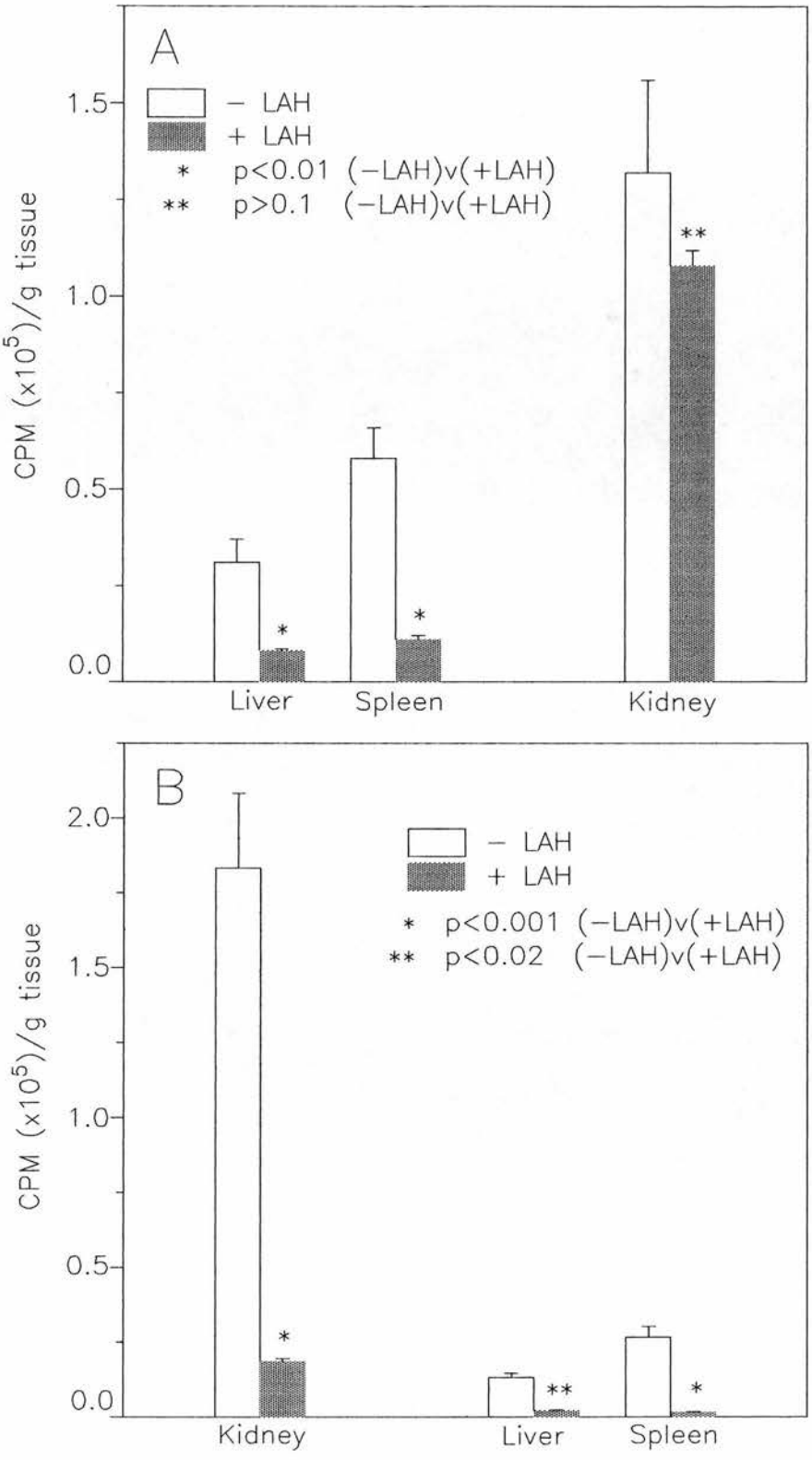


Figure 7A,B : Localisation of radioactivity in liver, spleen and kidney 60 minutes after a bolus i.v. injection of 50 anti−Xa U/kg of UFH (A) and enoxaparin (B). LAH was given 10 minutes prior to the heparin. Results presented are mean +/− SEM of 4 ☐ or 5 ▒ experiments.

undetectable levels (Figure 7B).

Administration of excess LAH reduced dramatically the amount of ^{125} I-enoxaparin that localised in the kidney of control rabbits ($p<0.001$, [-LAH v +LAH], Figure 7B). There was a trend towards a reduction in the amount of ^{125}I-UFH that localised in the kidney of control animals that received UFH and LAH compared with controls that received UFH alone, but this reduction only reached statistical significance in endotoxaemic animals ($p<0.05$, [-LAH v +LAH]). However, in absolute terms, pre-administration of LAH prevented far more ^{125} I-enoxaparin than ^{125} I-UFH from localising in the kidney.

Clearance of ^{125}I-labelled heparin

Pre-treatment of animals with LAH resulted in a significant prolongation of the elimination half-life of ^{125}I-labelled heparin. In control animals, the half-life of ^{125}I-UFH was dramatically prolonged by RES blockade, and was approximately 2.1 times longer in animals pre-treated with excess LAH compared with animals that received only UFH ($p<0.001$, Table 8), while the half-life of enoxaparin in the presence of LAH was only 1.3 times longer than in animals that received only enoxaparin ($p<0.01$, Table 8). Experimental endotoxaemia did not alter the effect of RES blockade on the clearance of ^{125}I-labelled heparin. The elimination half-life of ^{125}I-heparin in the presence of excess LAH was similar in endotoxaemic and control animals ($p=0.05$, UFH; $p=0.5$, enoxaparin; Table 8).

Loss of the RES as a route of heparin clearance had a greater effect on clearance of

Table 8 : Plasma half-lives (minutes) of heparin-derived radioactivity in control and endotoxaemic rabbits after 50 anti-factor Xa U/kg of heparin.

	Plasma half-life (minutes) *					
	Control			LPS-pretreated **		
Heparin	-LAH	+LAH §	p value	-LAH	+LAH §	p value
UFH	13.6 ± 1.2	29.2 ± 1.4 †	<0.001	16.7 ± 0.9	36.7 ± 2.8 †	<0.001
LMWH	23.1 ± 1.0	29.7 ± 1.6 ‡	<0.01	24.0 ± 0.5	27.7 ± 1.8 ‡	0.07

Values are mean ±SEM of 4 or 5 experiments. **† p=0.05 ‡ p=0.5**

*** Plasma half-lives ($t_{1/2}$) are determined by linear regression analysis of the semilogarithmic plot of plasma radioactivity v time.**

**** LPS was administered 2 hours before the heparin.**

§ Excess LAH was given as the RES blocking agent 10 minutes prior to the heparin.

UFH than enoxaparin, and the half-lives of the two heparins in control rabbits in the presence of RES blockade became similar ([29.2 ± 1.4] v [29.7 ± 1.6] minutes, p = 0.8, Table 8). However, this similarity in clearance rates in the presence of RES blockade was seen only in control animals as, unexpectedly, the elimination half-life of ^{125}I-UFH was longer than that of ^{125}I-enoxaparin in endotoxaemic animals ([36.7 ± 2.8] v [27.7 ± 1.8] minutes, p = 0.04). This is in contrast to clearance rates in the absence of RES blockade. Although UFH was cleared more quickly than enoxaparin in the absence of excess LAH, the clearance rates were not affected by experimental endotoxaemia and UFH was cleared more quickly than enoxaparin in both sick and

well animals (Table 8).

Quantification of urinary ^{125}I-heparin

The effect of RES blockade on urinary heparin excretion was not altered by experimental endotoxaemia, and so the results from control rabbits are presented here and are considered representative. Bladder urinary radioactivity in rabbits given LAH prior to UFH was 10 times greater than in the absence of LAH ($p<0.001$, Table 9), and was similar to levels in animals that received enoxaparin. In contrast, bladder urinary radioactivity in rabbits given LAH prior to enoxaparin was only 1.6 times greater than in the absence of LAH ($p<0.05$, Table 9).

Characterisation of urinary ^{125}I-heparin

The elution profiles of urine from control and endotoxaemic animals in the presence of RES blockade were similar, and results from control animals are considered representative. RES blockade did not alter the molecular weight profile of excreted ^{125}I-enoxaparin, as the elution profiles of urine from control animals given enoxaparin in the presence and absence of RES blockade were similar (Figure 8B). In contrast, the elution profile of urine from control animals given LAH prior to UFH was similar to that of the non-metabolised iodinated starting compound (Figure 8A).

Recovery of heparin ex vivo

Pre-administration of LAH resulted in a significant improvement in heparin recovery

Table 9 : Excretion of radiolabelled heparin in control rabbits in the presence and absence of RES blockade.

Heparin Type (50 anti-factor Xa U/kg)	Bladder Urinary Radioactivity * (CPM x 10^6) - LAH	+ LAH **	*p value*
UFH	0.14 ± 0.04	1.49 ± 0.07	$0.12.10^{-6}$
LMWH	2.28 ± 0.33	3.64 ± 0.37	0.02

Values are mean ± SEM of 3-5 experiments.

*** Bladder urinary radioactivity is the amount of radioactivity present in urine 60 minutes after a bolus i.v. injection of 50 anti-factor Xa U/kg of UFH or enoxaparin, and is expressed in absolute terms (CPM x 10^6).**

**** LAH was given as the RES blocking agent 10 minutes prior to the heparin.**

(Tables 10A-C). At each time point in the first 20 minutes after heparin administration, the plasma heparin concentration was approximately 30 to 50% higher in animals that received excess LAH before UFH or enoxaparin compared with those that received only heparin. However, the improvement in recovery was more marked in the middle and last tertiles following heparin administration, and most notably, plasma UFH concentrations in the last tertile were improved by preadministration of LAH from barely detectable levels to clinically significant concentrations (Figures 9A,B).

There was no difference in the extent of non-specific protein binding between the two heparins in control animals who had received excess LAH, as there was no significant

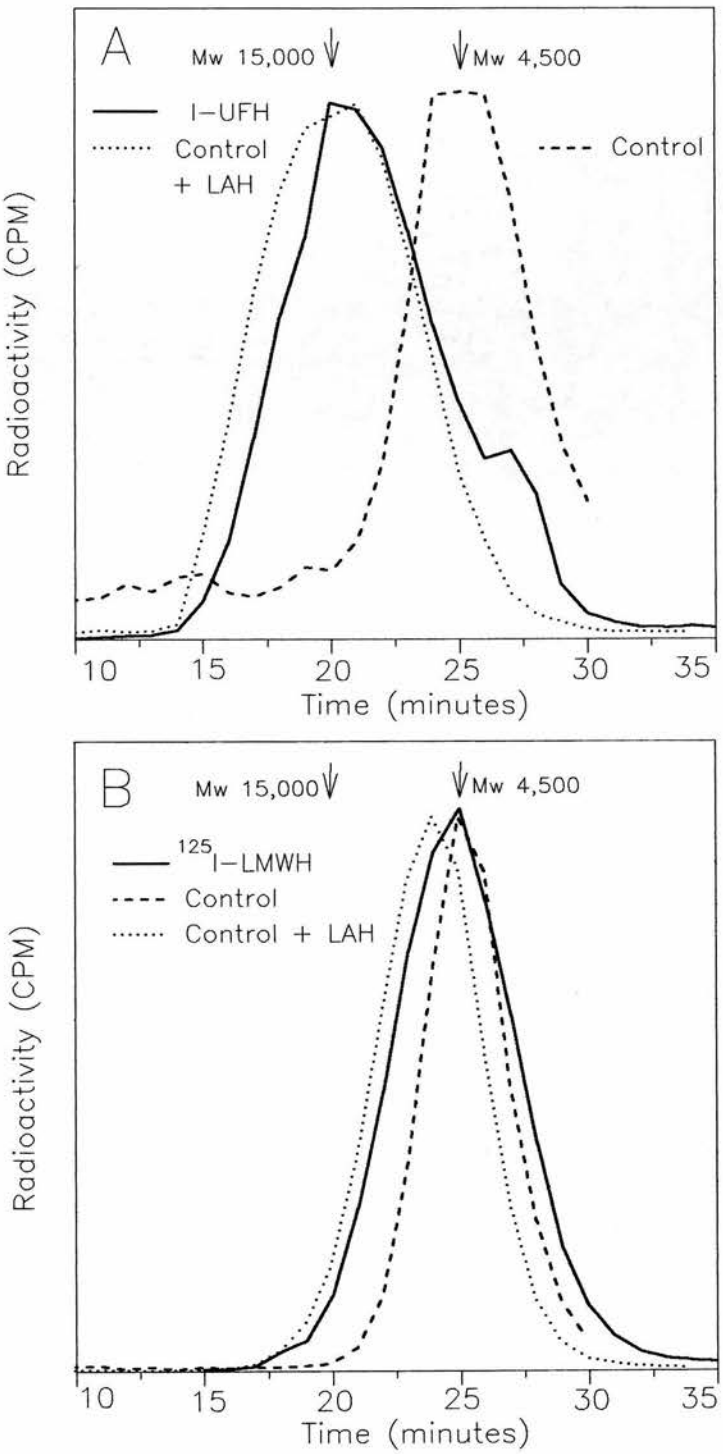


Figure 8A,B : HPLC profile of non–metabolised radiolabelled UFH —— (A) and enoxaparin —— (B) in rabbit urine, and radiolabelled UFH (A) and enoxaparin (B) in urine from control rabbits in the presence ····· and absence ----of LAH. LAH was given as the RES blocking agent 10 minutes prior to a bolus i.v. injection of 50 anti–Xa U/kg of heparin.

Table 10A : Plasma UFH concentrations in the presence and absence of excess LAH in control and endotoxaemic rabbits.

Time * (minutes)	Plasma UFH concentration (U/ml) ** Control - LAH	Control + LAH ‡	*p value*	LPS-treated † - LAH	LPS-treated † + LAH ‡	*p value*
5	0.43 ± 0.09	0.86 ± 0.09	0.01	0.54 ± 0.10	0.89 ± 0.09	0.04
10	0.45 ± 0.04	0.78 ± 0.02	<0.0001	0.45 ± 0.09	0.96 ± 0.07	0.004
15	0.39 ± 0.04	0.66 ± 0.03	0.001	0.42 ± 0.08	0.87 ± 0.06	0.004
20	0.32 ± 0.04	0.66 ± 0.02	<0.001	0.37 ± 0.08	0.82 ± 0.08	0.008

Values are mean ± SEM of 4 or 5 experiments.

*** Time (minutes) after a bolus i.v. injection of 50 anti-factor Xa U UFH/kg.**

**** The plasma UFH concentration (U/ml) was determined by measuring the anti-factor Xa activity of UFH in each plasma sample.**

† LPS was administered 2 hours prior to the UFH.

‡ Excess LAH (20-fold molar excess : UFH) was given 10 minutes before the UFH.

Table 10B : Plasma enoxaparin concentrations in the presence and absence of excess LAH in control and endotoxaemic rabbits.

Time * (minutes)	Plasma LMWH concentration (U/ml) ** Control - LAH	Control + LAH ‡	*p value*	LPS-treated † - LAH	LPS-treated † + LAH ‡	*p value*
5	0.63 ± 0.10	0.95 ± 0.04	0.04	0.51 ± 0.06	0.80 ± 0.07	0.03
10	0.52 ± 0.05	0.81 ± 0.08	0.02	0.51 ± 0.07	0.82 ± 0.14	0.04
15	0.45 ± 0.05	0.64 ± 0.03	0.02	0.54 ± 0.06	0.76 ± 0.08	0.07
20	0.43 ± 0.04	0.72 ± 0.05	0.004	0.44 ± 0.02	0.74 ± 0.09	0.02

Values are mean ± SEM of 4 or 5 experiments.

*** Time (minutes) after a bolus i.v. injection of 50 anti-factor Xa U enoxaparin/kg.**

**** The plasma LMWH concentration (U/ml) was determined by measuring the anti-factor Xa activity of enoxaparin in each plasma sample.**

† LPS was administered 2 hours prior to the enoxaparin.

‡ Excess LAH (20-fold molar excess : LMWH) was given 10 minutes before the enoxaparin.

Table 10C : Percentage increase in plasma heparin concentrations as a result of pre-administration of excess LAH in control and endotoxaemic rabbits.

Time (minutes) *	Increase (%) ‡ UFH control	UFH LPS †	enoxaparin control	enoxaparin LPS †
5	33.1 ± 7.1	46.3 ± 18.7	44.3 ± 2.0	43.0 ± 10.0
10	38.0 ± 2.9	51.2 ± 13.0	38.4 ± 8.1	42.5 ± 16.3
15	37.8 ± 5.4	50.5 ± 12.8	34.9 ± 5.8	35.3 ± 12.4
20	43.9 ± 2.7	51.1 ± 14.1	44.5 ± 6.8	36.8 ± 11.9

Values are mean ± SEM of 4 or 5 experiments.

*** Time (minutes) after a bolus i.v. injection of 50 anti-factor Xa U/kg of UFH or enoxaparin.**

† LPS was administered by bolus i.v. injection 2 hours before the heparin.

‡ The absolute plasma heparin concentration in the absence of LAH was subtracted from the concentration in the presence of LAH, and the difference was expressed as a percentage of the concentration in the absence of excess LAH, giving the % increase caused by LAH.

Note : in the presence of excess LAH, the absolute plasma heparin concentrations at each time point in the first 20 minutes after heparin administration were similar in all animals, irrespective of whether they received UFH, enoxaparin or LPS ($p>0.05$).

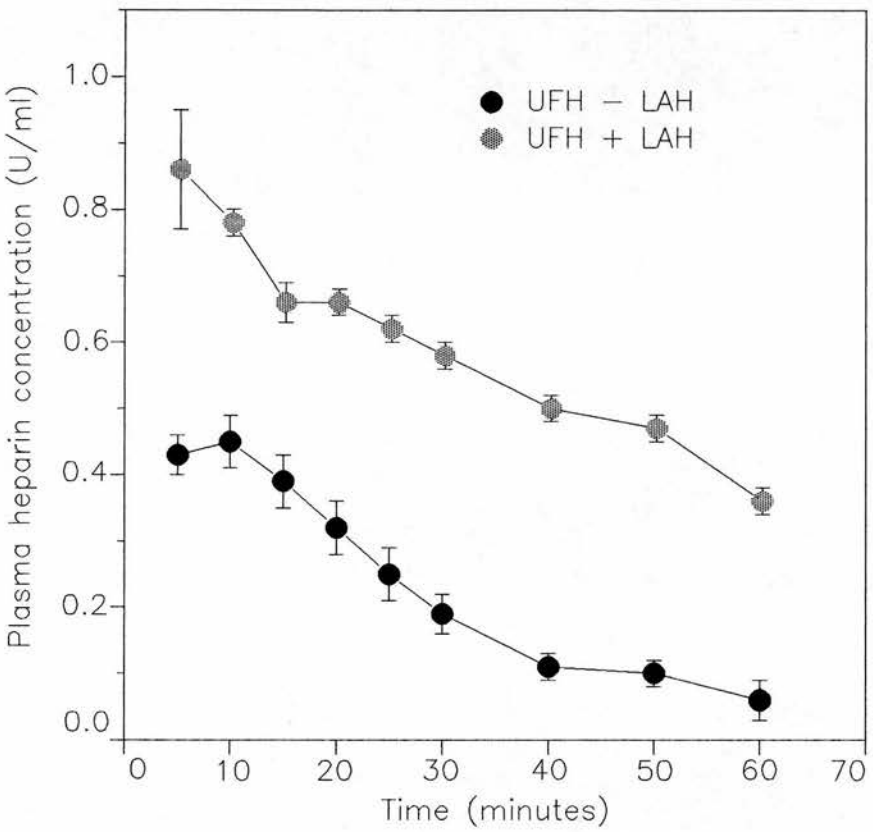


Figure 9A : Plasma heparin concentration (U/ml) in the first 60 minutes after a bolus i.v. injection of 50 anti−Xa U/kg of UFH to control rabbits in the presence ◍ and absence ● of RES blockade. LAH was given as the RES blocking agent 10 minutes prior to the UFH. Results presented are mean +/− SEM of 4 ◍ or 5 ● experiments.

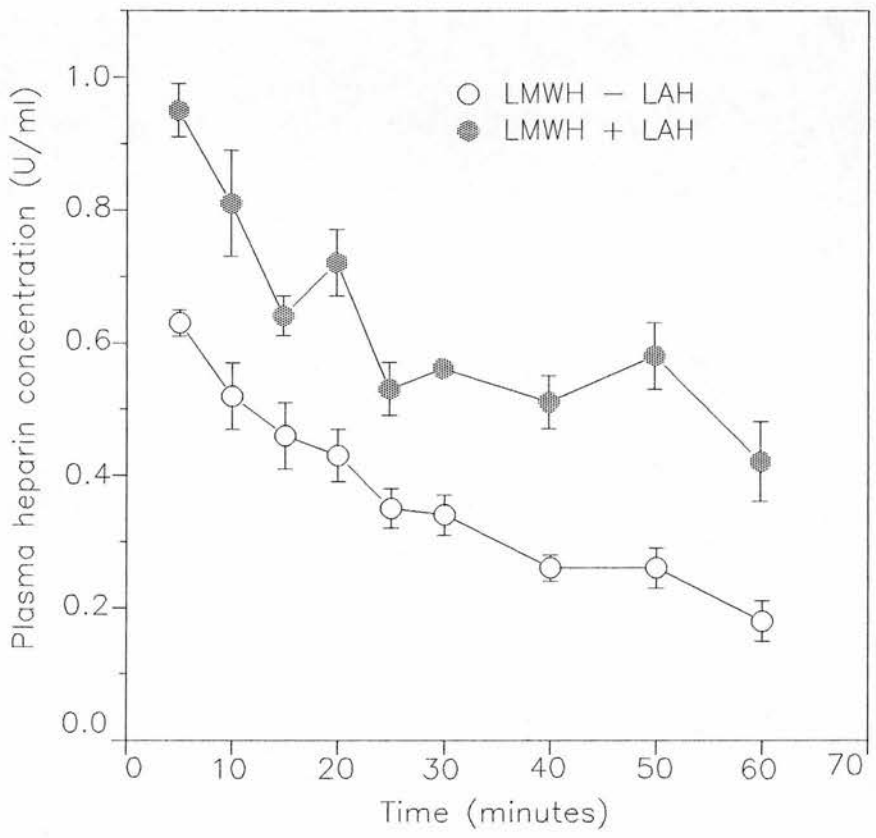


Figure 9B : Plasma heparin concentration (U/ml) in the first 60 minutes after a bolus i.v. injection of 50 anti−Xa U/kg of enoxaparin to control rabbits in the presence ● and absence ○ of RES blockade. LAH was given as the RES blocking agent 10 minutes prior to the enoxaparin. Results presented are mean +/− SEM of 4 ● or 5 ○ experiments.

difference in the improvement in recovery between UFH and enoxaparin, but the difference in extent of protein binding between the two heparin types became apparent in endotoxaemic animals (Table 10C). Moreover, in animals that received UFH, there was a trend towards greater improvement in recovery in endotoxaemic animals compared with controls, while the improvement in recovery in animals that received enoxaparin was similar in control and LPS-pretreated animals (Table 10C).

DISCUSSION

The clinical effectiveness of UFH is limited by factors which determine its pharmacokinetic and pharmoacodynamic characteristics. Of great importance is the marked variation in UFH dose-response that is seen in patients with thromboembolic disease (Hirsh et al, 1976). This variability is problematic and clinically important, because a strong relationship exists between clinical effectiveness and anticoagulant effect. Prospective studies have shown that the risk of suffering a recurrent venous thromoembolic event is 10 to 15 times greater in patients who fail to achieve therapeutic anticoagulation within 48 hours of initiation of heparin therapy (Hull et al, 1986; Basu et al, 1972) and patients who have suffered an acute myocardial infarction are 20 times more likely to develop a left ventricular mural thrombus if anticoagulation is inadequate (Turpie et al, 1989), and 6 times more likely to experience recurrent angina pectoris or a second myocardial infarction (Kaplan et al, 1987). Consequently, the anticoagulant response to UFH must be monitored carefully by a laboratory test and the dosage adjusted to produce a defined anticoagulant effect

as quickly as possible after initiation of therapy.

The limitations of UFH have been overcome by the development of the LMWHs, which have important differences in their plasma protein and endothelial cell binding characteristics when compared with UFH. LMWHs are fragments of commercial grade UFH produced by either chemical or enzymatic depolymerisation. Depolymerisation leads to some loss of the original catalytic activity (Jordan et al, 1982) with the ability to catalyse thrombin inhibition decreasing to a much greater extent than the ability to catalyse the inhibition of factor Xa (Andersson et al, 1979; Ofosu et al, 1990; Thunberg et al, 1979). The relative importance of anti-factor Xa and anti-IIa activities in exerting an antithrombotic effect remains unresolved. Some observations suggest that the most important action of UFH and the LMWHs in exerting their anticoagulant effects and possibly their antithrombotic effects occurs through their ability to inactivate thrombin (Ofosu et al, 1988, 1989, 1990; Béguin et al, 1988,1989). These observations are challenged by findings from experimental animals (Ofosu et al, 1990; Boneu et al, 1993) and clincial trials that suggest that both the anti-factor IIa and anti-factor Xa activities contribute to the antithrombotic effects of the LMWHs.

It would be clinically advantageous to devise an anticoagulant that not only retains the 1:1 anti-Xa : anti-IIa ratio of UFH and so has optimal antithrombotic activity but also has the recovery and clearance advantages of the LMWHs. This study has shown that the disadvantages of UFH can be overcome by the novel in vivo use of excess modified UFH which blocks the RES and binds non-specifically to plasma proteins,

resulting in improved recovery and a longer half-life. Nevertheless, the increase in the anticoagulant activity of UFH combined with the anticoagulant and antithrombotic effects of LAH in vivo may be offset by an increase in bleeding complications, and further studies are needed to assess the clinical applicability of this cheap and novel form of pharmacokinetic and pharmacodynamic manipulation.

LAH has been used in vitro and ex vivo to assess the extent of reversible heparin binding to non-specific plasma proteins (Young et al, 1992; Young et al, 1994). The modified UFH has dramatically reduced anticoagulant activity which can be attributed to a marked decrease in high-affinity AT III binding (Casu et al, 1986) such that it is practically devoid of anti-factor Xa activity. However, this study has shown that when administered in large amounts, LAH has significant anti-factor Xa activity. It is concluded that this is due to the anticoagulant activity that is mediated through a low affinity AT III binding site on the modified UFH which facilitates formation of a ternary LAH-AT III-Xa complex but results in a much reduced rate of enhancement of proteinase inhibition compared with high affinity heparin in keeping with the hypothesis of Olson and Björk (Olson and Björk, 1994). An anticoagulant effect will also be mediated through LAH-catalysed AT III-independent HC II activity (Tollefsen, 1989) but this will cause anti-factor IIa activity which will not interfere with the anti-factor Xa-based assessment of reversible protein binding used in this study.

In order to modify the clearance mechanism of UFH and so prolong its biological half-life, RES blockade was attempted. Various agents have previously been used as

RES blocking agents. Pretreatment with high doses of uni- or multilamellar vesicles will transiently reduce hepatic uptake of liposomes in the mouse (Kao and Juliano, 1981; Abra et al, 1980). Pretreatment with latex beads will slow hepatic clearance of compounds but does not have a significant effect on reduction of hepatic uptake per se (Kao and Juliano, 1981). ^{125}I-labelled aggregated albumin is known to be eliminated mainly by the RES (Benacerraf et al, 1957), but similarity of the radiolabel with the preparation used in clearance experiments prevented its use in this study. Compounds that are toxic to liver macrophages have also been used to block hepatic uptake of molecules, including methyl palmitate (Tanaka et al, 1975) and dextran sulphate (Patel et al, 1983) but the effect of these compounds on other clearance mechanism components is uncertain. In addition, because heparins and dextran sulphate are structurally and functionally similar, dextran sulphate is not a suitable RES blocking agent when studying glycosaminoglycan metabolism. Thus, the saturable nature of the cellular mechanism of UFH uptake was exploited by the novel use of excess LAH in vivo as a RES blocking agent. The chemically-modified UFH is produced by periodate oxidation followed by borohydride reduction. Although these chemical techniques cleave the C_2-C_3 bond of non-sulphated uronic acid residues, the molecular weight distribution, charge density, electrophoretic mobility and sulphate content of the parent molecule are preserved (Casu et al, 1986). Sulphated glycosaminoglycan receptors have been identified on the mouse macrophage cell membrane (Bleiberg et al, 1983), but the mechanism by which heparin binds to the receptor has not been described. This study provides evidence that heparin binding to

the macrophage is not mediated through the unique high-affinity pentasaccharide sequence necessary for AT III binding as this sequence is altered by the chemical process required to produce LAH. As less uptake of LMWH than UFH by the RES was found in this study, it is concluded that the macrophage receptor mechanism may be dependent upon the chain length, extent of sulphation or anionic charge of the molecule.

Earlier statements on the importance of the RES in UFH catabolism (Dawes and Pepper, 1979) are supported by the finding that in the presence of RES blockade, UFH was excreted unchanged compared with the starting radiolabelled material. This suggests that either UFH is degraded after uptake by the RES, or UFH must be metabolised by the RES before it can be degraded at a distant site.

In contrast to UFH, the main organ of localisation of enoxaparin was found to be the kidney. This finding is consistent with other reports on the excretion of LMWH (Boneu et al, 1987b; Palm and Mattsson, 1987b; Stehle et al, 1992). This study has extended these observations by demonstrating in vivo that the mechanism of renal clearance is associated with uptake of enoxaparin by renal cells, and it is proposed that a substantial proportion of LMWH that passes into the glomerular filtrate is taken up by renal cells before being excreted into the urine. When excess LAH was administered in vivo, uptake of enoxaparin by renal cells was dramatically reduced. The mechanism by which LAH prevented uptake of enoxaparin by renal tissue is unclear. LAH was administered in very high concentrations and it is possible that only the lower molecular weight moieties of LAH passed into the glomerular filtrate and

competed with enoxaparin for renal uptake. Alternatively, the competing molecules in the glomerular filtrate could have been metabolites of LAH that had been processed in reticuloendothelial cells.

Not only did administration of LAH in vivo improve heparin recovery, but the difference between recovery in the absence and presence of LAH in vivo increased over time. This time-dependence results from the combination of reduced non-specific plasma protein binding and altered clearance mechanisms. This study has previoulsy shown that the anticoagulant activity of heparin in plasma increases after administration of LAH in vivo as a result of displacement of heparin from circulating plasma proteins. Also, heparin uptake by the RES is prevented by preadministration of excess LAH, and so UFH clearance must now proceed by the slower renal route. Therefore, preadministration of LAH not only improves the biological activity of heparin in plasma but also facilitates increased duration of activity. As more UFH than enoxaparin is bound non-specifically to plasma proteins and the RES is the predominant route of UFH clearance, it follows that the effect of LAH in vivo on recovery will be greater with UFH than enoxaparin.

CHAPTER 5

DISCUSSION

Unfractionated heparin

UFH was discovered inadvertently in 1916 by McLean who, as a medical student, was looking for a coagulant produced by the liver (McLean, 1916). The mechanism by which UFH exerts its anticoagulant activity involving specific binding of UFH to the naturally-occurring serine proteinase inhibitor AT III (Brinkhous et al, 1939; Abildgaard, 1968; Rosenberg and Lam, 1979; Rosenberg and Bauer, 1994; Lindahl et al, 1979; Höök et al, 1976) has been discussed in the Introduction section of this thesis.

It is also known that UFH binds non-specifically to certain plasma proteins (Lane, 1989), including histidine-rich glycoprotein (Lijnen et al, 1983), PF4 (Holt and Niewiarowski, 1985), vWF (Sobel et al, 1991) and vitronectin (Preissner and Müller-Berghaus, 1987), with loss of anticoagulant activity.

The wide variation in anticoagulant response to UFH found among patients with thromboembolic disease (Hirsh et al, 1976) is clinically important because the effectiveness of UFH depends upon achieving a clearly defined anticoagulant response (Hull et al, 1986; Basu et al, 1972). As stated earlier, there is an increased risk of recurrent thromboembolic events in patients in whom anticoagulation is suboptimal (Hull et al, 1986; Turpie et al, 1989; Kaplan et al, 1987). The anticoagulant response to UFH requires careful monitoring by laboratory testing to ensure that the dosage may be adjusted to produce a defined effect, referred to as the therapeutic range.

Low molecular weight heparins

LMWHs have been developed over the past 20 years to enable the limitations of UFH, which have been described in the Introduction section of this thesis, to be overcome. LMWHs are as safe and effective as UFH for the prevention and treatment of venous thrombosis and pulmonary embolism both in hospital and at home (Prandoni et al, 1992; Hull et al, 1992; Levine et al, 1996; Koopman et al, 1996; Büller et al, 1997), and in the treatment of unstable angina (Cohen et al, 1997). Like UFH they exert their anticoagulant effect through interaction with AT III but only 15 to 25 percent of LMWH chains contain the unique pentasaccharide binding sequence (Harenberg, 1990). In addition, fewer than half of the chains are of sufficient length to bind to both AT III and thrombin (Jordan et al, 1980) and so they have greater activity against factor Xa than thrombin. It is of significance that non-specific protein binding of LMWHs is much less than of UFH and they have superior plasma recovery when administered at low doses. It has been found possible to administer safely weight-adjusted doses of the LMWHs for prophylaxis and treatment once or twice daily by subcutaneous injection without the necessity of monitoring by laboratory testing.

Variability in anticoagulant response

The factors responsible for the variation in heparin dosage requirments in patients with venous thromboembolism have not been identified, but mechanisms that determine heparin clearance and bioavailability have been implicated. The half-life of

UFH was observed to be shorter in patients with pulmonary embolism than in patients with venous thrombosis (Hirsh et al, 1976; Simon et al, 1978). It has been demonstrated that experimental pulmonary emboli rapidly become covered with a layer of platelets whereas the thrombi from which these emboli are derived are relatively platelet-free (Chiu et al, 1977), and the possibility was raised that the observed differences in heparin clearance in venous thrombosis and pulmonary embolism were due to release of antiheparin activity from platelets. The differences in clearance were confirmed in an experimental rabbit model of pulmonary embolism (Chiu et al, 1977) but interestingly, there was convincing evidence that platelets did not contribute to the shortened half-life in pulmonary embolism because heparin half-lives in thrombocytopenic and non-thrombocytopenic animals were identical. In addition, these experiments do not explain why variability in anticoagulant response is seen in venous thrombosis as well as in pulmonary embolism.

When heparin binds to plasma proteins other than AT III, its anticoagulant activity is reversibly neutralised (Lane, 1989). Young's group developed an ex vivo assay system which measured the proportion of heparin which is reversibly neutralised by binding to these plasma proteins (Young et al, 1992). They showed that reversible heparin neutralisation is a major determinant of the heparin dose required to achieve a therapeutic anticoagulant response, and that reversible binding to plasma proteins was more marked in patients with venous thromboembolism who had increased heparin requirements. This suggests that variability in dosage requirements is influenced by the concentration of heparin-neutralising proteins which could differ among patients with

venous thrombosis. The causative proteins were not identified, but the acute phase proteins were considered likely candidates. However, as the tendency to increased heparin requirements is present in the plasma of patients within a few hours of the onset of symptoms (Young et al, 1992), it follows that acute phase reactants cannot be responsible for increased heparin neutralisation as more than six hours must elapse from induction of inflammation before release of newly-synthesized acute phase proteins can occur (Koj, 1974; Castell et al, 1989; Hurlimann et al, 1966).

The purpose of this study was to determine the relative contributions of non-specific plasma protein binding and altered clearance to the variability in dose response to heparin and to the impaired anticoagulant response observed in some patients with thromboembolism. A reproducible in vivo model of heparin pharmacokinetics and pharmacodynamics was established using specific pathogen-free New Zealand White rabbits. These animals were bred and born in a sterile environment to ensure that they remain infection-free, as compared with farmyard rabbit strains traditionally used in research that are plagued with a wide variety of infective diseases (Harkness and Wagner, 1983; Flatt et al, 1974). It was considered important to use specific pathogen-free animals to ensure that control conditions were optimised and that control animals were not harbouring occult infection capable of inducing an acute phase response with its associated protein upregulation, which would potentially alter heparin recovery. By using these strains, alterations in heparin recovery could justifiably be ascribed to exogenous factors, such as the effect of endotoxin or differences between the heparin types. The rabbit was the preferred animal model. It is

the most commonly used animal in thrombosis models because its coagulation system is qualitatively similar to that of humans (Peyrou et al, 1997) and its size facilitates multiple blood sampling and post-mortem organ extraction, both of which are difficult in the rat and mouse.

The pharmacokinetics and pharmacodynamics of three doses of each heparin were studied. In order to have clinical relevance, a dose of 100 anti-factor Xa U/kg was chosen as this is similar to the amount of UFH that is given by bolus i.v. injection to patients before an i.v. heparin infusion is started, and the cellular mechanism of clearance is reported to be saturated by similar amounts (Caranobe et al, 1985; Boneu et al, 1987a). Two other doses were assessed which were fractions of the highest dose to ensure that a dose-response effect would be demonstrated if present in any of the characteristics under study.

In this study, the effect of acute inflammation on heparin clearance and reversible protein binding was examined. An established experimental model (endotoxin administration) was used to induce acute illness in rabbits. This model has been shown to increase the level of heparin binding proteins in rat plasma (Young et al, 1997) and to potentiate the development of venous thrombosis in rabbits (Bernat et al, 1994). As the tendency to increased heparin requirements may be present in patient plasma within a few hours of the onset of symptoms, a dose of endotoxin was used that produced major multisystem perturbations within 2 hours of administration (Mathison et al, 1988). Since hepatic acute phase protein mRNA does not increase significantly for 4 to 8 hours after acute phase induction and release of newly-synthesised acute

phase proteins occurs many hours after the onset of inflammation (Koj, 1974; Castell et al, 1989; Hurlimann et al, 1966), it is likely that the model of acute inflammation used in this study predominantly assessed the extent of reversible heparin binding to proteins that are released from storage sites in endothelial and other vascular cells as a response to endotoxin injury. Thus, heparin binding proteins such as PF4 (Rucinski et al, 1990; Maccarana and Lindahl, 1993), lactoferrin (Wu et al, 1995) and vWF (de Romeuf and Mazurier, 1993) may be released from endotoxin-activated platelets, neutrophils and endothelial cells, respectively, and a similar situation may be occurring in patients with thromboses who develop a reduced response to heparin within a few hours of onset of their symptoms. The presence of advanced endotoxaemia was determined by the clinical status of the rabbit and our animals exhibited signs of severe endotoxaemia within 2 hours of receiving a dose of LPS greater than that known to induce maximal tumour necrosis factor production (Mathison et al, 1988). It was not considered necessary to quantify markers of acute endotoxaemia such as interleukin-6 or tumour necrosis factor (Castell et al, 1989) to confirm the presence of advanced endotoxaemia because of the convincing clinical status of the animals.

The results of this study have shown for the first time that the phenomenon of impaired heparin recovery in vivo is caused by an increase in the concentration of heparin binding proteins. There was no evidence that increased heparin clearance contributes to the reduced heparin recovery observed in acute illness. Although the results in rabbits may be quantitatively different, they are likely to be qualitatively similar (Peyrou et al, 1997). This study suggests that variability in anticoagulant

response to heparin seen in patients is likely to be caused by variations in plasma protein binding rather than alterations in heparin clearance. In contrast to UFH which showed a significant reduction in recovery in endotoxin-treated animals, the recovery of LMWH was unaffected by acute endotoxaemia. Therefore, although the study supported the contention that LMWH binds to a modest extent to proteins in normal plasma, the results showed that unlike UFH it does not bind to the proteins released from endotoxin-activated platelets, endothelial cells or neutrophils. These findings provide an explanation for the observation that patients treated with LMWH exhibit a less variable dose-response than those treated with UFH (Handeland et al, 1990).

Clearance kinetics

Heparin clearance can be assessed either by measuring the rate of disappearance of its anticoagulant activity from plasma or by estimating the rate of disappearance of tracer amounts of radiolabelled heparin administered with excess non-labelled heparin. The latter method is based on the assumption that radiolabelled heparin is cleared from the circulation in a manner identical to that of non-labelled anticoagulant. The major anticoagulant effect of UFH and LMWH results from their ability to bind with high affinity to AT III through a unique pentasaccharide binding sequence present on only a minority of heparin chains. Heparin chains lacking this sequence have low affinity for AT III and are devoid of anticoagulant activity in vitro. Despite the absence of an anticoagulant effect, they still have antithrombotic activity as UFH has greater antithrombotic action than high affinity fractions alone (Barrowcliffe et al, 1984).

Therefore, the disappearance of the anticoagulant activity of heparin from the intravascular compartment only assesses the clearance of fractions with high affinity for AT III and excludes those fractions devoid of anticoagulant activity but which still have antithrombotic activity and are clinically relevant. It is of greater clinical relevance to measure the disappearance of radioactivity from plasma using tracer amounts of radiolabelled heparin. The combined rate of clearance of all the different heparin fractions is assessed by this technique because the ability of the radiolabel to bind to the heparin chain does not depend upon chain length or the presence of a specific binding sequence. For these reasons, tracer amounts of radiolabelled heparin were used to determine the clearance kinetics of various doses of UFH and enoxaparin.

In this study a similar clearance pattern was seen for each dose of heparin administered, and this was true for each heparin type, in both control and endotoxin-treated animals. At each dose, an initial rapid disappearance phase of about 5 minutes' duration occurred which is consistent with an equilibration, or distribution, phase of clearance when mixing of the drug in plasma, binding to plasma proteins and distribution to another compartment (such as the endothelium) takes place, and data from previous studies supports the occurrence of an initial rapid distribution phase (de Swart et al, 1982; Olsson et al, 1963; Glimelius et al, 1978; Mahodoo et al, 1977). For both UFH and enoxaparin, distribution was followed by a slower elimination phase, and for each dose the half-life of UFH was shorter than that of enoxaparin, results which support earlier work (Boneu et al, 1987a, 1987b). When plasma

radioactivity was plotted semilogarithmically as a function of time, the linearity of the relationship suggested that elimination was proceeding by a first-order process (Gillies et al, 1986). This finding was similar for each heparin type at all doses, both in control and endotoxin-treated animals, but is inconsistent with results from previous studies of heparin kinetics. These studies did initially report that UFH disappeared exponentially after i.v. administration (Olsson et al, 1963; Estes et al, 1969; Bjornsson et al, 1982); these first-order kinetics were confirmed by others (Perry et al, 1974) except Simon, who found that UFH removal in normal subjects was nearly linear, indicative of a zero-order process (Simon et al, 1978). Moreover, most investigators agreed that UFH half-life increased with dosage when the anticoagulant was administered by bolus i.v. injection (Bjornsson and Levy, 1979; Olsson et al, 1963; Bjornsson et al, 1982; Estes, 1970), although first-order kinetics dictate a constant half-life. De Swart observed that the curve that describes the disappearance of a large dose of heparin had a concave-convex shape, suggestive of a model that combines a saturable (and exponential) and a non-saturable (and linear) mechanism of clearance (de Swart et al, 1982). Boneu confirmed these observations and proposed that the saturable mechanism had two main cellular components, endothelial and reticuloendothelial cells, and that the non-saturable route of clearance was renal (Boneu et al, 1987a, 1987b). There are several explanations for the differences in clearance curves between this study and previous works. The majority of clearance kinetics in Boneu's studies were based on the disappearance of anticoagulant activity from plasma which is a measurement of the fractions with high affinity for AT III. In

contrast, the clearance curves in this study were determined from clearance of radiolabelled heparin. The clearance curve of ^{125}I-labelled heparin is an amalgam of the clearance curves of all the different heparin fractions, including those with both high and low affinity for AT III which are known to be cleared at different rates (Caranobe et al, 1986). This method of determining clearance was chosen as it is of far greater clinical relevance than anticoagulant-based methods because it estimates the half-life of exogenous antithrombotic material in plasma. In these circumstances differences between results from the studies of Boneu et al and those from this investigation are not perhaps surprising.

An alternative explanation may be obtained by consideration of the cellular mechanism of clearance. Although the cellular mechanism is saturated at doses of between 75 and 100 anti-factor Xa U/kg in other systems (Caranobe et al, 1985; Boneu et al, 1987a), in this model it remained the dominant mechansim of clearance at all the doses studied. This study found that the clearance of antithrombotic activity was exponential for both heparin types, but that the half-life of UFH was relatively dose-dependent while the half-life of enoxaparin was relatively dose-independent and so consistent with a first-order process. However, as UFH is thought to be cleared predominantly by the cellular mechansim which follows first-order kinetics (de Swart et al, 1982; Boneu et al, 1987a) and low molecular weight heparin by the renal mechansim which follows zero-order kinetics (Boneu et al, 1987b), theoretically the half-life of UFH should be dose-independent and that of enoxaparin should increase with increasing dose. Although previous reports support both clearance mechanisms and processes,

no explanation for these inconsistencies have been given. Based on previous knowledge and the findings of this study, it would appear that basic physiological and anatomical data is of paramount importance and must be incorporated into the construction of a biologically meaningful kinetic model, while computerised curve fitting programmes take secondary importance. Based on the findings in this study, the following is proposed as an explanation of heparin clearance kinetics and is not inconsistent with previous observations (Boneu et al, 1987a, 1987b; Goudable et al, 1986; Bara et al, 1985; Caranobe et al, 1985; Bergqvist et al, 1983; Aiach et al, 1983; Thomas and Merton, 1982; de Swart et al, 1982; Dawes and Pepper, 1979). The pharmacokinetic properties of UFH and LMWH determine that they are cleared from the blood by a combination of a saturable and nonsaturable mechanism and that the relative contributions of these two mechanisms depend upon the dose and type of heparin injected. The terms 'saturable' and 'non-saturable' describe (respectively) the limited and unlimited capacity that the components of each mechanism have for heparin, and do not relate to the kinetics of the clearance processes. At low doses within the therapeutic range, UFH is cleared primarily by the efficient saturable, or cellular mechanism. As the dose increases, proportionally more UFH is cleared by the slower, nonsaturable renal mechanism until the cellular mechanism is saturated and the predominant route of clearance is renal. The half-life of UFH is dose-dependent because although the kinetics of each clearance mechanism are exponential, the rates of clearance differ between the two mechanisms. UFH is cleared by a combination of the two mechanisms and as the dose increases, proportionally more UFH is cleared by

the slower renal mechanism, and so the half-life of UFH increases until it approximates that of LMWH at a dose of about 100 anti-factor Xa U/kg, when the cellular mechanism is saturated and the renal route becomes the dominant clearance route. In contrast, LMWH is cleared primarily by the non-saturable mechanism. The contribution of the saturable mechanism to its clearance is negligible, irrespective of the dose administered, as the cellular mechanism is saturated by very low doses of LMWH. At therapeutic doses, the clearance of UFH by the cellular route is quicker than the clearance of LMWH by the renal route, explaining why after comparable doses the half-life of UFH is shorter than that of LMWH.

Clearance components

The components of the cellular mechanism of clearance have not been fully determined, but there is good evidence that the RES, the endothelium and the kidneys all play a role in the cellular, or metabolic clearance of UFH. In contrast, the major component of LMWH clearance is the kidney and minimal clearance occurs by the metabolic pathway (Monkhouse, 1954; Piper, 1947; Caranobe et al, 1985; Boneu et al, 1987b). The liver and spleen were implicated in UFH metabolism when intravenously administered UFH was found to accumulate in these organs before being released back into the circulation fully desulphated and lacking biological activty but retaining the molecular weight of the starting material (Dawes and Pepper, 1979). The liver was confirmed as a metabolic site when it was observed that the biological effect of UFH persisted for longer in hepatectomised rats (Losito et al, 1981).

Receptors for sulphated glycosaminoglycans are found on the mouse macrophage cell membrane (Bleiberg et al, 1983), and evidence exists that hepatic uptake of UFH may be scavenger receptor-mediated involving liver macrophages and sinusoidal endothelial cells (Stehle et al, 1992). Although O-desulphamidase activity has yet to be described, a heparin N-desulphamidase has been demonstrated in various mammalian tissue including rat spleen (Friedman and Arsenis, 1974). Hepatectomy reduces the amount of desulphated glycosaminoglycan present in rat plasma after i.v. administration, but desulphated material persists at a level greater than expected from splenic desulphation (Wells and Dawes, 1995). This suggests that there is another site of desulphation in vivo. Specific endothelial binding sites for heparin have been identified (Barzu et al, 1985; Vannucchi et al, 1988) where bound glycosaminoglycans are internalised and degraded to small fragments. Endothelial cells also produce fully desulphated carbohydrate chains from a variety of radiolabelled glycosaminoglycans (Dawes and Pepper, 1992) and it has been suggested that endothelial non-scavenger receptor binding sites play a role in heparin uptake (Stehle et al, 1992). It therefore appears that the RES and at least one other site, probably the vascular endothelium, are responsible for desulphation of UFH.

In contrast, although a small amount of LMWH is eliminated by metabolic clearance, the major route of LMWH elimination is by renal excretion. At the doses used in this study, about 15% of the total dose of injected radiolabelled enoxaparin had been excreted within 1 hour of administration while in certain circumstances, up to 70% of intravenously administered radiolabelled LMWH can be eliminated by the kidneys

(Palm and Mattsson, 1987b).

The saturable nature of the metabolic pathway of elimination has been confirmed by this study. For both UFH and LMWH, the amount of radiolabelled anticoagulant that accumulated in the liver and spleen fell as the dose of excess unlabelled heparin increased. Although proportionally less radiolabelled heparin accumulated in the organs as the dose of heparin increased, the amount of radiolabelled heparin administered at each dose was kept constant. Assuming that the clearance of radiolabelled heparin is the same as that of the unlabelled molecule, this means that the amout of unlabelled heparin that accumulated in the organs was similar at all 3 doses. Therefore, the liver and spleen were unable to significantly increase their uptake of heparin despite more being available because their capacity for uptake was finite, or saturable, at the doses under study. In contrast, renal uptake of each heparin was unlimited. Because the amount of radiolabelled heparin was held constant, the lack of change in the amount of radiolabelled heparin that accumulated in the kidneys meant the amount of unlabelled heparin that accumulated increased with increasing dose. This means that at the doses under study, renal capacity for heparin uptake is unlimited and non-saturable. As expected, the RES was saturated by significantly smaller amounts of enoxaparin than UFH, in agreement with the minimal role proposed for the liver and spleen by Caranobe (Caranobe et al, 1985) and by Boneu (Boneu et al, 1987b) in the elimination of LMWH.

Surprisingly, comparable amounts of radiolabelled UFH and enoxaparin were associated with the kidney, which is inconsistent with previous work that suggested

greater accumulation of LMWH than UFH (Palm and Mattsson, 1987b). Different types of LMWHs were however used in the two studies. Several chemical and enzymatic methods are used to produce the LMWHs which result in a heterogeneous group of compounds with marked differences in their physico-chemical characteristics (chemical structure, nature of the salt, molecular weight, molecular weight distribution range and charge density pattern). The difference in renal accumulation of the LMWHS may have been caused by differences in their physico-chemical characteristics.

The results from this study suggest that UFH and LMWH are dealt with by the kidney in different ways. Studies comparing radioactivity levels in urine and kidneys have shown that LMWH elimination from plasma is by renal excretion into urine, while elimination of UFH is mediated via metabolic, or cellular clearance with some accumulation occurring in the renal tissue (Boneu et al, 1987a, 1987b; Palm and Mattsson, 1987b). The identity of the renal cells which accumulate UFH is unknown, and their function has not been fully determined. The kidneys have been proposed as the site of degradation of the glycosaminoglycan once it has been fully desulphated (Dawes and Pepper, 1979) but although a heparin-degrading endoglycosidase has been described in rat spleen (Höök et al, 1977), a similar enzyme has not been found in renal tissue to confirm that this is indeed the site of UFH degradation. There is some evidence that the kidney also plays a role in heparin desulphation but promising in vitro findings have not been supported by in vivo studies using nephrectomised rats (Comper et al, 1994; Wells and Dawes, 1995). The major role of the kidney in the

elimination of LMWH was confirmed in this study as about ten times more radiolabelled enoxaparin than UFH was excreted into the urine. There was no evidence that enoxaparin had undergone degradation prior to excretion whereas the molecular weight profile of excreted UFH was similar to that of enoxaparin. A possible explanation can be obtained if it is considered that only those heparin fractions that have been completely desulphated may undergo subsequent degradation. As hepatic uptake of LMWH is minimal, LMWH will remain sulphated and so will not undergo degradation prior to excretion. This proposition is supported by the observation that when large doses of UFH are administered intravenously to rabbits, the majority of the anticoagulant cannot be desulphated as hepatic uptake is saturated, and the UFH is excreted intact (Dawes and Pepper, 1979). In this study, the in vivo use of LAH as a RES blocking agent had a similar effect, confirming the importance of RES uptake in heparin catabolism. Finally, as with any experimental method, the lack of detectable degraded enoxaparin may have resulted from lack of adequate sensitivity of the detection method rather than through the absence of very low molecular weight enoxaparin fractions per se. This is considered to be unlikely but if so the amount of undetected degraded enoxaparin would be so low as to be of minimal clinical significance.

Acute experimental endotoxaemia did not alter uptake of either heparin type by the RES. This implies that the scavenger receptor-mediated uptake of heparin by the macrophage cell system remains functionally intact and is not compromised by endotoxaemia. This is in contrast to other cells whose functions are altered during

endotoxaemia, including endothelial cells, platelets, neutrophils, monocytes and fibroblasts (Billiau and Vandekerckhove, 1991). This has clinical implications as the heparin-macrophage scavenger receptor interaction may account for several antithrombotic properties of heparin which are independent of its anticoagulant activity. In vitro and in vivo studies have shown that stimulation of macrophages by heparin promotes profibrinloytic activity, possibly through increased secretion of urokinase type plasminogen activator (Falcone, 1989; Markwardt and Klöcking, 1977; Vinazzer et al, 1982; Bacher et al, 1992; Harenberg et al, 1990), and may play a role in low density lipoprotein metabolism and endothelial cell/macrophage interactions (Srinivasan et al, 1991; Lindstedt et al, 1992; Murata et al, 1988; Harenberg et al, 1989). Thus, heparin-RES cell interactions appear to play a complex and important role in the biology of the vessel wall with implications for physiological and pathological mechanisms, and the findings of this study suggest that this role is preserved during the insult of acute endotoxaemia and its associated challenge to vessel wall integrity (Morrison and Ulevitch, 1978; Billiau and Vandekerckhove, 1991)

LAH - a therapeutic tool?

LAH was developed in order to provide a method of assessing the extent of reversible heparin neutralisation by non-specific plasma protein binding in vitro and ex vivo (Casu et al, 1986; Young and Hirsh, 1990). The anti-factor Xa activity of LAH is <1.0 U/mg, but it has significant anticoagulant activity when administered in large

amounts because anti-factor Xa activity can be mediated through a low affinity binding site on the modified UFH (Olson and Björk, 1994), and anti-factor IIa activity can occur through LAH-catalysed, AT III-independent HC II (Tollefsen, 1989).

UFH has several pharmacokinetic and pharmacodynamic characteristics which hinder its clinical usefulness. The LMWHs have similar anticoagulant efficacy compared with UFH but are free from the characteristics of UFH that have restricted its clinical use. Their main limitation is related to production-associated expense, with LMWH costing 3 to 4 times more than a comparable dose of UFH. It follows that if the advantages of the LMWHs could be bestowed upon UFH, the latter anticoagulant would become a far more attractive anticoagulant at a fraction of the cost of LMWH. Thus, factors determining the bioavailablity and clearance of UFH were manipulated by the novel in vivo administration of excess LAH prior to UFH. In the presence of excess LAH, UFH could no longer be cleared by the RES as cellular uptake mechanisms were saturated by LAH. This suggests that scavenger receptor-mediated uptake of glycosaminoglycans by macrophages did not occur through the pentasaccharide binding sequence which is altered in the production of LAH. As chain length differences are responsible for differences in heparin-endothelial cell binding characteristics (Barzu et al, 1984), uptake by RES cells may be similarly dependent upon chain length and/or degree of sulphation, neither of which is affected by the LAH production process (Young and Hirsh, 1990). A significant increase in bladder urinary radioactivity confirmed that the renal mechanism becomes the dominant clearance method when the RES is saturated (Boneu et al, 1987a). The necessity for

metabolic clearance prior to UFH catabolism was validated in this study when radiolabelled fractions of a similar molecular weight profile to the non-metabolised iodinated starting material were excreted in the presence of excess LAH. Preadministration of LAH caused a significant improvement in plasma anticoagulant recovery by providing an alternative substrate for non-specific protein binding. This improvement was time-dependent with the difference in recovery in the absence and presence of LAH increasing over time because with LAH preadministration, the increased amounts of anticoagulantly-active UFH were eliminated by the slower renal clearance mechanism. Confirmation of the clinical benefits of LAH resulting from this improvement in recovery requires further experiments to ensure that the improvement is not accompanied by an increased bleeding tendency.

It is of interest to note that excess LAH caused a significant decrease in the amount of radiolabelled enoxaparin that accumulated in renal tissue but did not alter accumulation of UFH, which suggests that UFH and LMWH interact with different cell types in the kidneys. Renal cells involved in LMWH uptake may have a higher affinity for longer than shorter chain lengths, and so preferentially take up LAH which blocks LMWH uptake. In contrast, cells involved in UFH uptake may only interact with heparin which has the unique pentasaccharide binding sequence, and so preadministration of LAH will not alter these cells' uptake of UFH. Similar experiments involving preadministration of LAH but using LAH labelled with a different isotope to that of UFH and enoxaparin would provide useful information to help explain the above findings.

CHAPTER 6

CONCLUSION

Since its first clinical application over fifty years ago, UFH has remained until recently the preferred anticoagulant for the prophylaxis and treatment of a wide range of conditions. However, despite its extensive clinical application, its pharmacokinetic and pharmacodynamic characteristics remain poorly understood. Its clinical use is hindered by its poor bioavailability after subcutaneous injection, and this feature coupled with its short half-life determine that it is administered therapeutically by continuous i.v. infusion. There is wide variation in the anticoagulant response to a standard dose of UFH and the most extreme example of this is heparin resistance, when large amounts of UFH are required to produce a therapeutic anticoagulant response. For this reason, the anticoagulant response must be monitored closely by laboratory testing, and the dose adjusted to achieve an anticoagulant response within the therapeutic range. Factors responsible for the variability in dose response have not been identified, but reversible heparin neutralisation by non-specific protein binidng and increased heparin clearance in disease states have been thought to be implicated.

In contrast, the LMWHs offer several advantages over UFH. They can be administered once or twice daily by subcutaneous injection which allows them to be administered in the out-patient setting. They also produce a more predictable response to weight-adjusted doses which obviates the need for labratory monitoring. They have been shown to be as safe and effective as UFH in the prevention and treatment of venous thrombosis, pulmonary embolism and unstable angina, and recently have been used in the out-patient management of uncomplicated venous thrombosis and pulmonary embolism.

In order to improve our understanding of heparin bioavailability and clearance, and in

an attempt to identify the factors responsible for variation in UFH dose response, a reproducible in vivo model of heparin pharmacokinetics and pharmacodynamics was established in the rabbit. This has demonstrated that the phenomenon of impaired heparin recovery is caused by an increase in concentration of heparin binding proteins. The extent of reversible protein binding is substantially less with LMWH than UFH, and in contrast to UFH which showed a significant reduction in recovery in acute experimental endotoxaemia, LMWH does not bind to heparin binding proteins that are released in reponse to endotoxin as its recovery was unaffected by acute endotoxaemia. The study findings suggest that these heparin binding proteins are unlikely to be acute phase reactants but are more likely to be proteins released from activated platelets, endothelial cells or leucocytes. There was no evidence that increased heparin clearance contributes to the reduced heparin recovery observed in acute illness.

The novel in vivo application of LAH produced significant improvements in UFH recovery and half-life. This has important clinical implications as the benefits of LMWH could be gained for a fraction of its cost by using low affinity and unfractionated heparins in combination. Although the results in rabbits may be quantitatively different from those in humans, they are likely to be qualitatively similar, and so it is probable that the variable anticoagulant response to heparin seen in patients with venous thrombosis is caused by variable plasma protein binding rather than alterations in heparin clearance.

CHAPTER 7

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CHAPTER 8

MATERIALS AND METHODS

MATERIALS

UFH with Mw_{Av} 15,000 and anti-factor Xa activity of 160 U/mg and the LMWH enoxaparin with Mw_{Av} 4,500 and anti-factor Xa activity of 100 U/mg (Mw_{Av} and specific activities as stated by the manufacturer) were obtained from Rhône-Poulenc Santé, France. SHPP was supplied by Pierce Chemical, Canada. Carrier-free ^{125}I was obtained from Dupont, USA. LPS obtained from *Salmonella minnesota* R595 by phenol-chloroform-petroleum-ether extraction (Sigma Chemical Co., USA) was suspended in 1x sterile phosphate buffered saline (2mg/ml) and solubilized by sonication. Human AT III (Cutter Biological, USA) and Sepharose (Pharmacia, Canada) were used to prepare AT III-Sepharose affinity chromatography columns. Progel TSK HPLC columns and PD-10 columns were purchased from Pharmacia, Canada and Supelco, Canada, respectively. Specific pathogen free New Zealand White male rabbits were used for all animal experiments (Charles River, Montreal). Topical anaesthesia was achieved with EMLA cream (Astra Pharmaceutical Inc, Canada) prior to insertion of indwelling intra-arterial and intravenous Angiocaths (Becton Dickinson, USA) to which male luer lock plugs (Codan Medlon Inc, USA) were attached. Butterfly cannulae used in euthanasia were obtained from Venisystems, Abbott, Canada. Radioactivity quantification was carried out using a gamma counter (Clinigamma LKB 1272, Fisher Scientific, Canada). Anti-factor Xa assays were performed on the ACL automated coagulation analyser (Instrumentation Laboratory, Canada) using the Stachrom heparin kit (Diagnostica Stago, Wellmark Diagnostics, Canada).

METHODS

Preparation of ^{125}I-heparin

UFH was reacted with SHPP (Dawes and Pepper, 1979) and the product was labelled with ^{125}I based on the method of Greenwood (Greenwood et al, 1963). Commercial sodium heparin (10mg) was reacted with an excess of non-iodinated SHPP (10mg) in 5ml of 0.05 M sodium borate (pH 9.2) at 4^0 C for 20 hours. Unreacted ester, at this stage hydolysed to the acid, was removed by gel filtration in water on Sephadex G-25. The material eluted at the void volume was chromatographed on protamine-agarose to isolate the biologically active heparin derivatives. These were desalted, lyophilised, and 300μg aliquots were labelled with ^{125}I-Na. Twenty-five μL of ^{125}I-Na (1μL/0.1mCi) were added to 300μg of UFH-SHPP (1mg/ml dH_2O) followed by the oxidising agent chloramine-T solution (10μL of [5 mg chloramine/ml 0.4 M PO_4 buffer] solution). 0.4 M PO_4 buffer was prepared by adding buffer B (5.44 g KH_2PO_4/ 100ml dH_2O) to 500ml buffer A (28.43g $Na_2HPO_4 .7H_2O$/500ml dH_2O) until pH 7.4 was reached. The reaction was stopped 10-15 seconds after addition of chloramine-T with 20μL sodium metabisulphite (21mg NaS_2O_5 /3 ml 0.4 M PO_4 buffer, pH 7.4). The reaction was then quenched with 100μL sodium iodide solution (10mg NaI/5ml 0.4 M PO_4 buffer, pH 7.4) and unreacted ^{125}I-Na was removed by gel filtration in halide-free buffer (0.4 M PO_4 buffer, pH 7.4) on Sephadex G-25 (10ml bed volume). Equilibration of the gel was carried out with 0.4 M phosphate buffer, pH 7.4. The elution pattern (which was reproducible) was determined by gamma-counting 10μL samples from each 500μL eluate, and a non-radioactive discard fraction (2.5 ml) was

followed by the ^{125}I-UFH peak (1.5ml). In a typical process, approximately 20% of the ^{125}I-Na was incorporated and the iodinated UFH had a specific activity of 0.2 mCi/mg. The biological activity (as measured by anti-factor Xa assay) and molecular weight distribution (as measured by gel filtration) of the radiolabelled UFH were unchanged relative to the unlabelled starting material. The iodinated UFH was stored at -70^0 C in siliconised microfuge tubes. Enoxaparin was iodinated in an identical manner.

The iodinated heparin was reseparated if it was used in experiments more than two weeks after the initial iodination. The material was reseparated by gel filtration in phosphate buffered saline on Sephadex-G25 (10ml bed volume). 10μL samples from each 500μL eluate were gamma-counted, and a non-radioactive discard fraction (2.5ml) was followed by the ^{125}I-heparin peak (1.5ml). The specific activity of the reseparated iodinated heparin was then calculated prior to its use, and any unused iodinated material was again stored in siliconised microfuge tubes at -70^0 C.

Preparation of LAH

LAH was prepared from unfractionated porcine mucosal heparin (157 USP units/mg, Sigma Chemical Co., USA) by controlled periodate oxidation and borohydride reduction according to the method of Casu (Casu et al, 1986) with minor modifications (Young and Hirsh, 1990). UFH (500mg) was oxidized by sodium periodate in an aqueous medium at 4^0 C for 24 hours. The reaction was stopped by addition of ethylene glycol and dialysed against distilled water. The oxidized heparin

was reduced by the addition of sodium borohydride and the solution was adjusted to pH 3.0 to destroy excess borohydride and quickly readjusted to pH 7.0 to minimize acidic conditions. After a second overnight dialysis against distilled water, the LAH solution was adjusted to 1.0 M NaCl by the addition of solid salt. The product was precipitated with 3 volumes of absolute ethanol, recovered by centrifugation (2,000 x g at 4^0 C for 90 min) and air-dried.

In order to remove any remaining traces of high affinity material, the LAH was subjected to affinity chromatography on an AT III-Sepharose column. Human AT III (AT III; 726 units) and CNBr-activated Sepharose were used to prepare the column according to the manufacturer's instructions. The column (50ml bed volume) was equilibrated with starting buffer (0.05 M Tris/HCl, pH 7.4 containing 0.15 M NaCl). Approximately 60mg LAH was dissolved in 10ml starting buffer and pumped on to the column followed by another 90ml starting buffer. The salt concentration was switched to buffered 2.0 M NaCl and a further 90ml pumped through the column to elute any high affinity heparin. The concentration of the resultant low affinity fraction was measured by the protamine sulphate turbidimetric assay (Hatton et al, 1978). For comparative purposes, 10mg UFH was subjected to affinity chromatography in a similar manner. The flow-through material was recovered and re-applied to the re-equilibrated column. This procedure was repeated for a total of 3 times and the resultant low affinity fraction recovered and its concentration measured by the protamine sulphate assay. Gel filtration chromatography was performed on Sephadex

G-150 columns (Pharmacia). The samples (0.5ml) were adjusted to 1.0 M NaCl and applied to the column (0.9 x 55cm) equilibrated with the same salt concentration. One ml fractions were collected at a flow rate of 9 ml/h. The fractions were monitored by determination of uronic acid by the carbazole reaction (Bitter and Muir, 1962). For comparative purposes UFH was subjected to gel filtration chromatography in a similar way.

The molecular weight distribution of LAH was within the range encompassed by UFH, and the anticoagulant activities of LAH were essentially identical to that of LAH separated from UFH by repeated affinity chromatography. Although the AT III-dependent inhibition of factor Xa activity by LAH was almost completely abolished, LAH retained anti-factor IIa activity through the AT III-independent, HC II pathway. The anti-factor Xa activity of LAH was <1.0 U/mg, and the anti-factor IIa activity was 20 U/mg.

Animal Studies

All procedures were carried out in accordance with the recommendations of the Canadian Council on Animal Care for the care and use of experimental animals (Canadian Council on Animal Care, 1993b), and approved by the Animal Rights and Ethics Committee at McMaster University, Ontario, Canada. Specific pathogen free New Zealand White male rabbits (2.5 - 3.0 kg) were used for all experiments. Newly-acquired animals were allowed to acclimatise for a minimum of 6 days. The rabbits were housed individually in stainless steel cages with a single-floor area of 0.50 m^2,

and 6 animals were contained in each holding room. The holding room temperature was maintained at 16-20^0 C with a relative humidity of 40-50%, 10-20 air changes/hour and a light cycle of 12 hours' artificial light in each 24 hour period. The animals received 75-100g of pelleted commercial feed daily with a protein content of 14%. Autoclaved tin cans were provided as a source of environmental enrichment. Experiments were performed in an area separate from the holding room. One hour before insertion of indwelling i.v. and intra-arterial cannulae, the dorsal aspect of each ear was plucked and a liberal application of EMLA topical anaesthetic cream (2.5% lidocaine / 2.5% prilocaine) was placed over the central artery and marginal vein of each ear, and covered. Animals were placed in restrainers and allowed to settle before cannulae insertion. Control animals remained in the restrainers throughout the experiment until euthanasia was completed. Animals that received LPS were placed in the restrainers initially to facilitate insertion of cannulae and administration of agents. They were then placed on a cushioned area of the floor and were nursed by the handler throughout the experiment.

a) Control animals. Each rabbit was placed in the animal restrainer and allowed to settle. EMLA cream was removed with 70% isopropylalcohol. A 22G Angiocath was placed in the left central ear artery and secured, and a male luer lock plug was attached to the distal end before the cannula was flushed with 1ml 0.9% NaCl through the injection plug. The process was repeated for the right marginal ear vein. All sampling and injecting was performed through the injection plugs via the arterial and venous indwelling cannulae. Immediately before and after i.v. administration of study

material, a bolus i.v. injection of 1ml 0.9% NaCl was given to ensure patency of the cannula and to clear any residual injected material from the cannula. The animals were given a bolus i.v. injection into the marginal ear vein of either 20, 50 or 100 anti-factor Xa U/kg of UFH or enoxaparin in 1.5ml 0.9% NaCl to which a tracer amount (10uCi, <2 anti-factor Xa U) of the corresponding ^{125} I-heparin had been added. Five animals were used at each dose. The total amount of radioactivity administered was determined in the gamma counter before injection (Appendix 1). After the mixture had been injected, the empty syringe was weighed. Immediately before and at various times (1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 40, 50 and 60 minutes) up to 1 hour after injection, blood samples were collected from the central ear artery into buffered 3.8% sodium citrate (9 vol blood : 1 vol citrate). Immediately before sampling, a bolus i.v. injection of 1ml 0.9% NaCl was given via the central ear artery to confirm patency of the cannula, and 1ml blood (dead space volume) was withdrawn from the cannula and discarded. The blood samples were placed in siliconised microfuge tubes and put immediately on ice, and the weights of the full and empty syringes were recorded. Within 1 hour of sampling, PPP was prepared by centrifugation (1600 x g at room temperature for 15 minutes). After 60 minutes, a 23G butterfly cannula was placed in the left marginal ear vein and the animal was killed with a bolus i.v. injection of 3 ml Euthanyl (sodium pentobarbital, 240 mg/ml). The animal was removed carefully from the restrainer and a brief external examination of the heart was made. The animal was then placed on the post mortem table in the dorsal recumbency position with the limbs extended as far to the sides as possible. The abdominal and thoracic cavities were

entered in turn through midline incisions. Representative tissue samples were taken from the liver (right, anterior middle and left lobes), spleen and both kidneys. The lungs and spleen were inspected for signs of haemorrhage and congestion before samples were removed. The bladder wall was punctured under direct vision (20G needle), bladder urine was aspirated, the bladder urinary volume was recorded and 4 x 1ml urine samples were placed in siliconised microfuge tubes. The tissue samples were air-dried and placed in polypropylene tubes, and the tissue samples and 3 of the 4 urine samples were stored at -70^0 C prior to analysis.

b) LPS-treated animals. Initial dose-finding experiments were performed to determine the optimal dose of LPS required for the rapid induction of clinically-advanced endotoxaemia (Table 11). After a bolus i.v. injection of LPS, the mean arterial pressure was recorded at regular intervals through a pressure transducer attached to an indwelling cannula in the left central ear artery and a constant record of the animal's clinical condition was kept, paying particular attention to respiratory rate and effort, appearance of conjunctivae, and the behavioural pattern of the animal. Major organs were examined macroscopically at post mortem. The optimal dose of LPS was found to be 10 μg/kg. Rabbits were then pre-treated with LPS before receiving either UFH or LMWH. After cutaneous anaesthesia had been secured and cannulae placed (as above), rabbits received a bolus i.v. injection of LPS (10 ug/kg in 0.9% NaCl, final volume 1.5ml) into the right marginal ear vein. Two hours later, they were given 1 of the 3 doses of UFH or LMWH with the corresponding ^{125} I-heparin added (10uCi, <2 anti-factor Xa U) in 1.5ml 0.9% NaCl as a single bolus i.v. injection

Table 11 : details of LPS dose-finding experiments.

LPS #1 : 5μg/kg

Time (minutes)	0	+16	+32	+46	+60	+77	+93	+122	+153	+183	+210	+265	+275	+295	+325
MAP (mmHg)	77	70	66	50	37	40	50	40	35	25	30	N/R	32	25	N/R
Clinical Condition	Well	→	→	Laboured respiratory effort; tachypnoea; injected conjunctivae; intermittent lassitude; crying		→	→			Tachypnoea; intermittent crying; localising to back of cage; injected conjunctivae; frequency of micturition; lassitude ++					

At post mortem : heavy, diffusely haemorrhagic lungs, congested liver, swollen spleen. N/R : not recorded

LPS #2 : 10μg/kg

Time (minutes)	0	+15	+30	+45	+60	+75	+90	+105	+120	+135	+150	+165	+180
MAP (mmHg)	80	83	85	84	76	83	87	84	70	66	40	50	40
Clinical Condition	Well	→	→	→	→	Occasional whine; increasingly lethargic and withdrawn; increasing frequency of micturition				Withdrawn ++; poor and shallow resp effort; flaccid		Fit x1	Resp. arrest; †

At post mortem : heavy, diffusely haemorrhagic lungs, swollen spleen. † : died

LPS #3 : 10μg/kg

Time (minutes)	0	+15	+30	+45	+60	+75	+90	+130	+145	+160	+180
MAP (mmHg)	70	86	88	84	76	82	NR				

Clinical Condition	Well	→	→	→	Increasingly irritable	→	Fitted after pre-MAP saline flush; ++ irritable → MAP recording stopped	Markedly withdrawn; extensor posturing; irritable, drowsy; fitted x1

At post mortem : lungs - heavy, with scattered punctate heamorrhages and diffuse haemorrhage esp of lower lobes; swollen spleen.

LPS #4 : 10μg/kg

Time (minutes)	0	+15	+30	+45	+60	+75	+90	+125	+165	+180	+230
MAP (mmHg)	70	84	94	94	94	88	60	100	110	104	†

Clinical Condition	Well	Excitable before being placed in restrainer; well	→	→	→	Irritable ++; assuming lateral position when resting; ophisthotonic	Withdrawn ++; extended, flaccid; no response to pain

At post mortem : macroscopically normal. **N/R : not recorded** **† : died**

into the marginal ear vein. Blood, tissue and urine samples were collected as for the control animals. Five animals were used at each dose.

c) RES blockade. Sixteen rabbits received a bolus i.v. injection of LAH (20-fold molar excess : UFH or LMWH in 0.9% NaCL, in final volume 1.5ml) into the right marginal ear vein. Ten minutes later, 8 received a bolus i.v. injection of 50 anti-factor Xa U/kg of UFH with ^{125}I-UFH added (10 μCi, <2 anti-factor Xa U) in 1.5ml 0.9% NaCl into the marginal ear vein. The same number received a bolus i.v. injection of LMWH with ^{125}I-LMWH added (10 μCi, <2 anti-factor Xa U) in 1.5ml 0.9% NaCl. Four of each group of 8 animals were treated with LPS (as above) 110 minutes before LAH administration. Blood, tissue and urine samples were collected as for the control animals. Similar experiments were performed to evaluate heparin recovery and clearance when excess LAH was given after heparin. Eight rabbits received a bolus i.v. injection of 50 anti-factor Xa U/kg of UFH with ^{125}I-UFH added (10 μCi, <2 anti-factor Xa U) in 1.5ml 0.9% NaCl into the right marginal ear vein. The same number received a bolus i.v. injection of 50 anti-factor Xa U/kg of LMWH with ^{125}I-LMWH added (10 μCi, <2 anti-factor Xa U) in 1.5ml 0.9% NaCl. Four of each group of 8 animals were treated with LPS (as above) 120 minutes before heparin administration. Fifteen minutes after heparin administration, they received a bolus i.v. injection of LAH (20-fold molar excess : UFH or LMWH in 0.9% NaCl, final volume 1.5ml) into the marginal ear vein. Blood, tissue and urine samples were collected as for the control animals.

d) Anticoagulant activity of LAH in vivo. The in vivo anticoagulant activity of LAH given in 20-fold molar excess to a representative dose of UFH was assessed. Two rabbits received a bolus i.v. injection of LAH (20-fold molar excess : UFH, assuming 50 anti-factor Xa U UFH/kg had been administered) into the right marginal ear vein. One rabbit was treated with LPS (as above) 120 minutes before LAH administration. Fifteen minutes before and 10 minutes after LAH administration, they received a bolus i.v. injection of 1.5ml 0.9% NaCl into the same vein. Blood samples were collected immediately before and at various times (5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60 and 70 minutes) after LAH injection. After 70 minutes, the animals were killed (as above). The same experiments were repeated using LAH in 20-fold molar excess to LMWH, assuming 50 anti-factor Xa U LMWH/kg had been administered.

Ethanol precipitation : determination of optimal conditions for estimation of free ^{125}I in plasma samples

A stock solution of ^{125} I-UFH in 0.85M saline was prepared to an estimated specific activity of 2.10^2 CPM ^{125} I-UFH/µL 0.85M saline. Twenty µL of ^{125}I-UFH stock solution were added to 1ml 0.85M saline in triplicate, the gamma radioactivity emitted in 1 minute was counted and the mean value was calculated. Background radioactivity was the mean gamma radioactivity emitted in 1 minute from 4 x 1ml 0.85M saline, and the mean net radioactivity of the ^{125} I-UFH stock solution samples was calculated by subtracting the background radioactivity from the measured radioactivity of the stock solution. Twenty µL of ^{125} I-UFH stock solution were added to 80µL NRS,

100μL 0.85M saline and 600μL 100% ethanol (3 volumes ethanol : 1 volume ^{125} I-UFH/NRS/saline) in triplicate, left overnight at 4^0 C then centrifuged (1600 x g at room temperature for 15 minutes). 500μL supernatant were added to 0.85M saline (1:1) and the gamma radioactivity emitted in 1 minute was counted. The experiments were repeated using increasing amounts of excess unlabelled UFH instead of NRS, and the mixtures were allowed to precipitate at 4^0 C for 24 hours prior to centrifugation at 1600 x g at room temperature for 30 minutes.

The conditions required for maximal precipitation of bound ^{125} I were as follows :

1 vol plasma : 1 vol 0.85M saline : 1 vol excess unlabelled UFH (1mg UFH/100μl 0.85M saline): 9 vol 100% ethanol, precipitated at 4^0 C for minimum of 16 hours then spun at 1600 x g at room temperature for 30 minutes.

Pharmacokinetics

For each animal, the percentage of the injected dose of radioactivity present in 1ml plasma was plotted as a function of time after transformation of radioactivity into logarithmic values. The distribution and elimination half-lives ($t_{1/2}$ α and $t_{1/2}$ β) were determined by linear regression analysis (Appendix 1).

Quantification of tissue radioactivity

The tissue samples were stored in polypropylene tubes at -70^0 C prior to analysis. They were thawed at room temperature and the tubes were weighed on an electronic balance (Oertling, Avery Berkel, Canada) in the presence and absence of the tissue

sample. The weight of the tissue sample was the difference between the full and empty polypropylene storage tube. The gamma radioactivity emitted in one minute by the tissue (gross CPM) was counted. The machine background radioactivity was calculated from the mean CPM of 4 x 1ml 0.85M saline samples; this value was subtracted from the gross CPM of each tissue sample to give the CPM net of machine background radioactivity (net CPM).

Tissue radioactivity was expressed either in absolute terms (as counts per minute per gram tissue : CPM/g tissue), or in relative terms (as the percentage of the total dose of radioactivity injected per gram tissue : % total dose/g tissue) (see 'Quantification of urinary radioactivity' below).

Tissue-associated radioactivity was calculated using the following methodology :

i) Radioactivity per gram tissue (**CPM/g tissue**) : $\frac{\text{net CPM of tissue sample}}{\text{weight of tissue samples}}$

ii) With multiple samples from 1 organ, mean radioactivity per gram tissue (**mean CPM/g tissue**) : $\frac{\text{sum of i (CPM/g tissue) for all samples}}{\text{number of tissue samples}}$

iii) Tissue radioactivity per gram tissue as percentage of total dose of radioactivity injected (**% total dose/g tissue**) : $\frac{\text{i (CPM/g tissue) or ii (mean CPM/g tissue)}}{\textbf{Total Dose Injected}} \times 100$ (Figure 1B, Appendix 1)

To assess if measured radioactivity in tissues was indicative of tissue-associated radioactivity per se or merely a measure of radioactivity in blood trapped within tissues, it was assumed that tissue-associated radioactivity was secondary to radioactivity in trapped blood and expected values were calculated using established

blood volumes of tissues from control and LPS-treated animals (Mathison and Ulevitch, 1979) and measured plasma radioactivity levels at the time of tissue extraction. The measured values (Y axis) were plotted against the calculated values (X axis) (Phillips, 1992a) and the correlation coefficient was determined by linear regression analysis (Figure 10), where r = slope of the regression of the Y variable on the X, and is calulated from :

$$r = \frac{\Sigma xy}{NS_x S_y}$$

where :
r = Pearson coefficient
Σxy = sum of the cross products of deviations form the means of X and Y distributions
N = number of such products
S_x and S_y : standard deviations of the two distributions

There was no correlation between predicted and measured tissue-associated radioactivity values in the liver, spleen or kidney, confirming that the method for quantifying tissue radioactivity accurately measured tissue-associated radioactivity per se.

Quantification of urinary radioactivity

Four x 1ml urine samples were placed in siliconised microfuge tubes after removal from the bladder at post mortem. Three of the 4 samples were stored at -70^0 C prior to determination of the molecular weight characteristics of the excreted radiolabelled heparin. Samples from the fourth aliquot were used to determine the amount of radioactivity excreted in urine. The total amount of radioactivity in urine was

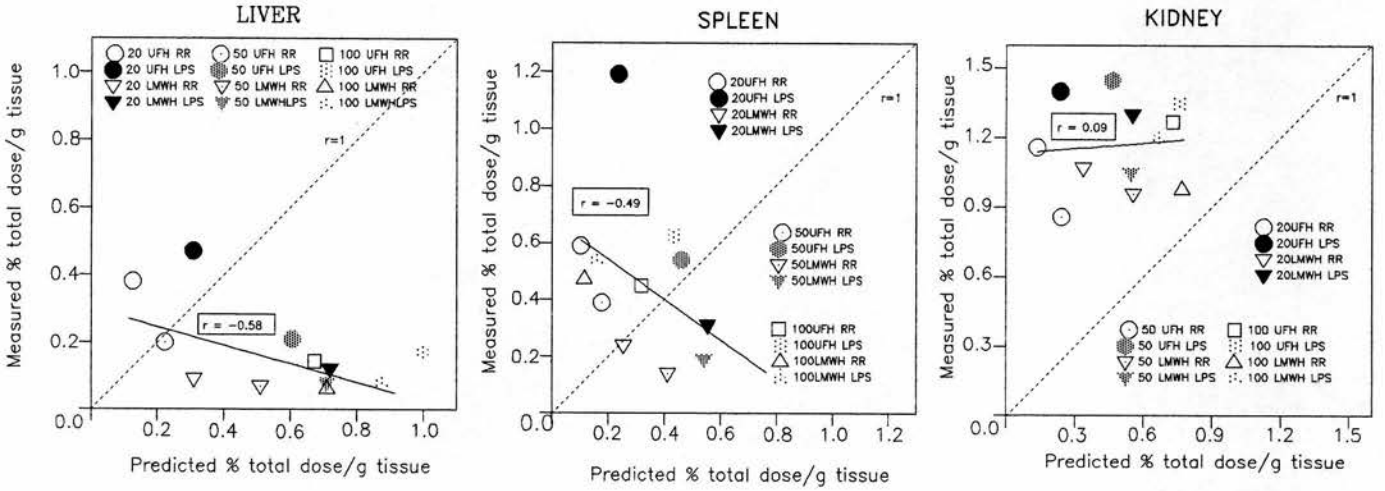

Figure 10 : Relationship between measured and predicted values for localisation of radioactivity in liver, spleen and kidney 60 minutes after a bolus i.v. injection of various doses of UFH and LMWH to control and LPS–treated rabbits.

determined by adding 100μL urine to 900μL 0.85M saline, in duplicate. The amount of radioactivity from free ^{125}I in urine was determined by ethanol precipitation of bound ^{125}I-heparin; 100μL urine were added to 100μL 0.85M saline, 100μL excess unlabelled heparin (1mg/100μL 0.85M saline) and 900μL 100% ethanol, in duplicate. All of the urine samples were stored with the plasma samples at 4^0 C for 48 hours. The ethanol precipitation samples were then centrifuged (1600 x g at room temperature for 30 minutes) and 500μL supernatant was added to 500μL 0.85M saline. The gamma radioactivity emitted in one minute from each sample (gross CPM) was counted and the mean gross CPM of the duplicate samples was calculated. The machine background radioactivity was calculated from the mean CPM of 4 x 1ml 0.85M saline samples; this value was subtracted from the mean gross CPM of each pair of samples, giving the CPM net of machine background radioactivity (net CPM). Bladder urinary radioactivity was expressed either in absolute terms as bladder urinary radioactivity (CPM), or in relative terms as the percentage of the corrected total dose injected (% total dose).

In animals that did not receive LAH in vivo, the calculated values for the total injected doses of radioactivity (Appendix 1) in control and endotoxin-treated animals were the same, and so comparisons of tissue and urinary radioactivity values between these groups were expressed in relative terms. However, the calculated values for the total injected doses of radioactivity in animals that received LAH in vivo were different to those in animals that did not receive LAH, and so comparisons of tissue and urinary radioactivity values between these groups were expressed in absolute terms.

The bladder urinary radioactivity was determined using the following methodology :

i) **Total radioactivity** (from free and bound ^{125} I)**/100μL urine** : net CPM/100μL urine.

ii) **Radioactivity from free ^{125} I/100μL urine** : (net CPM of 500μL subsample of ethanol pecipitation solution) x 2.4. A correction factor of 2.4 (1200÷500) is used because the 1200μL final volume ethanol precipitation solution contained 100μL urine, and 500μL of the 1200μL was counted.

iii) **Radioactivity from bound ^{125} I/100μL urine** : (i - ii).

iv) **Bladder urinary radioactivity** (radioactivity from bound ^{125} I/ bladder urinary volume): iii(radioactivity from bound ^{125} I/100μL urine) x $\frac{\text{bladder urinary volume (μL)}}{100}$

v) **Total dose injected corrected for free ^{125} I (CPM bound ^{125} I)** : CPM total (Figure 1B, Appendix 1) - percentage of CPM total that was non-precipitable (Figure 1B, Appendix 1).

vi) **Bladder urinary radioactivity as percentage of total dose injected (CPM bound ^{125} I):**

$$\frac{\text{iv (bladder urinary radioactivity)}}{\text{v (total dose injected)}} \times 100$$

All plasma, tissue and urine samples were counted together.

Chromatographic analysis of radiolabelled heparin in urine

HPLC analysis of urine was performed using a liquid chromatograph (System Gold,

Beckman Instruments Inc., Canada) equipped with a model 126 solvent delivery system and a manual injector. A TSK G3000 SWXL 30cm x 7.8mm column was used with a mobile phase of 0.5M Na_2SO_4 and a flow rate of 0.5ml/min; ΔOD at 205nm was recorded. Preliminary experiments showed that the chromatographic profiles of high and low molecular weight particles (most likely calcium carbonate and triple phosphate crystals) present in rabbit urine were indistinguishable from those of buffered UFH and LMWH. Ethanol precipitation, heat denaturation and treatment with trypsin all failed to remove the particulate matter. Therefore, urine containing bound ^{125} I-heparin was applied to the column and the gamma radioactivity in each eluate was measured. The radioactivity-time profile of eluted rabbit urine was then compared with the chromatograms of buffered iodinated UFH and LMWH in rabbit urine, and heparin molecular weight standards. Rabbit urine was precipitated in absolute ethanol (1 vol urine : 3 vol 100% ethanol at 4^0 C for 48 hrs) and the supernatant was discarded after centrifugation (1600 x g at room temperature for 30 minutes). The residual pellet was resuspended in 0.85M saline, 100μL suspension was applied to the column and 0.5ml fractions were collected. The gamma radioactivity emitted from each fraction in 1 minute (CPM) was counted. The presence of heparin in the suspension and the absence of heparin in the supernatant were confirmed by Alcian Blue testing. 2μL samples of suspension and supernatant were spotted on to filter paper (1x Whatman's) and air-dried. The paper was then stained in 0.1% Alcian Blue (10 ml of [0.1g Alcian Blue/ 10ml dH_2 0] + 90 ml glacial acetic acid) for 10 minutes, destained in deionised water and air-dried. Control samples were also applied

(2μL UFH [10mg/ml 0.9% NaCl], LMWH [10mg/ml 0.9% NaCl], 0.9% NaCl and rabbit urine). The presence of intense blue stain indicative of heparin was confirmed in the UFH and LMWH samples, absence of stain from the NaCl and rabbit urine samples was noted, and the presence or absence of the intense blue stain of heparin was recorded in the test urine samples. The radioactivity-time profiles of the starting materials were determined. A sample of iodinated UFH or LMWH in rabbit urine (1 vol ^{125} I-heparin : 3 vol urine) was precipitated in absolute ethanol (1 vol ^{125} I-heparin/urine : 3 vol 100% ethanol at 4^0 C for 48 hrs, followed by centrifugation at 1600 x g at room temperature for 30 minutes, supernatant discarded) and the pellet was resuspended in buffer (0.5M Na_2SO_4). 200μL suspension was applied to the column and the gamma radioactivity emitted from each 0.5ml fraction in 1 minute (CPM) was measured. The radioactivity-time profiles of the non-metabolised and excreted iodinated heparins were compared and the approximate molecular weights of the heparin fractions in the eluates containing peak radioactivity were determined from the molecular weight - retention time standard curve constructed from molecular weight standards obtained from the manufacturer (Rhône-Poulenc Santé, Canada).

Anticoagulant assays

Plasma heparin concentrations were measured as anti-factor Xa activity (Teien and Lie, 1977) using the Stachrom heparin kit which had been adapted for use on the ACL automated coagulation analyser. The assay is carried out with an excess of AT

III to ensure that any existing deficiency of this protein is compensated for. The test principle is based on the *in vitro* inhibition of factor Xa by AT III-heparin complexes :

$$\text{AT III} + \text{heparin} \rightarrow [\text{AT III-heparin}]$$

$$[\text{AT III-heparin}] + \text{Xa (known excess)} \rightarrow [\text{AT III-heparin-Xa}] + \text{Xa (residual)}$$

$$\text{Xa (residual)} + \text{CBS 31.39} \rightarrow \text{pNA}$$

The quantity of paranitroaniline (pNA) released at 405 nm is inversely proportional to the amount of heparin (UFH or LMWH) present in the plasma. A calibration curve is constructed from standards prepared by adding UFH or LMWH in vitro to pooled PPP from healthy rabbits (to a final concentration of 0.8U heparin/ml plasma). Two controls corresponding to 0.8 and 0.4 U heparin/ml plasma (neat and 1:2 dilution of the appropriate standard) were assayed with each batch of test samples. 25µl standards, controls or test plasmas were added to 25µl AT III solution and 450µl diluted buffer, and incubated for 2 minutes at 37^0 C. 25µl factor Xa, 25µl chromogenic substrate CBS 31.39 and 25µl 50% acetic acid were then added in turn. After each addition, the samples were mixed and incubated at 37^0 C for 30 seconds, and finally the ΔOD at 405nm of the sample read against a blank was recorded.

A 3 point calibration curve was constructed on linear graph paper by plotting the heparin level for each calibration point (0.8U heparin/ml plasma, 1:2 dilution of [0.8U heparin/ml plasma] and a sample blank of buffer solution [1 vol 10-fold concentrated Tris EDTA buffer, pH 8.4 : 10 vol ddH_20]) against the corresponding ΔOD value.

The heparin levels (U/ml) of the test samples were deduced from the calibration curve. Assay linearity was observed for UFH levels between 0.0 and 0.8 U/ml and for LMWH levels between 0.0 and 1.0 U/ml. Pooled rabbit PPP was used to dilute samples into the range of the standard curve. UFH and LMWH levels less than 0.05 U/ml (which was the detection limit of the assay) had no meaningful significance.

Displacement of heparin bound non-specifically to plasma proteins

The anti-factor Xa activity of each plasma sample was assayed to quantify the amount of unbound UFH or LMWH present. LAH (2.1μL of 0.585 mg/ml PBS, <1anti-factor Xa U/mg) was then added to each assay sample to displace anticoagulantly-active heparin which was bound to plasma proteins other than AT III, and the resultant anti-factor Xa activity assessed. This concentration of LAH represents addition of LAH in at least 20-fold molar excess to the estimated molar concentration of UFH or LMWH in each sample and has been shown previously to be in excess of the amount required to displace all of the protein-bound heparin (Young et al, 1993a). The observed increment in anti-factor Xa activity cannot be accounted for by the LAH since it has minimal anti-factor Xa activity (<1 U/mg). Therefore, the amount of anticoagulantly-active heparin bound to plasma proteins other than AT III is represented by the difference in anti-factor Xa activity before and after the addition of LAH.

Heparin recovery

Analysis of recovery was limited in the majority of animals to the first 20 minutes

(first tertile) after heparin administration because there was no significant recovery of the lowest dose of UFH after this time, and the half-lives of the doses of UFH and LMWH under study determined that major changes in recovery occurred within the first 20 minutes of administration. In animals that received LAH in vivo, absolute plasma heparin concentrations were also assessed over the 60 minutes following heparin administration, and were compared with similar values obtained from animals that received only heparin.

The amount of unbound heparin in each plasma sample was quantified by anti-factor Xa assay. The assay was repeated in the presence of excess LAH to quantify the amount of free and plasma protein-bound heparin present, or total heparin. The anti-factor Xa activity was measured in plasma drawn at 5, 10, 15 and 20 minutes after heparin administration. Recovery was calculated by measuring the area under the plasma concentration versus time curve ($AUC_{0\text{-}20}$), using the trapezoidal rule method (Gillies et al, 1986) where the plasma concentration versus time curve is depicted as a number of trapezoids by drawing straight lines between each adjacent plasma concentration value (Figure 11). Although the upper side of the trapezoid is a curve, as the time interval (width of trapezoid) is small, it can be assumed that the upper side is a straight line and the error incurred is slight. The area of each trapezoid is calculated from (Figure 11) :

$$\text{area} = \frac{(C_2 + C_1)}{2} \times (t_2 - t_1)$$

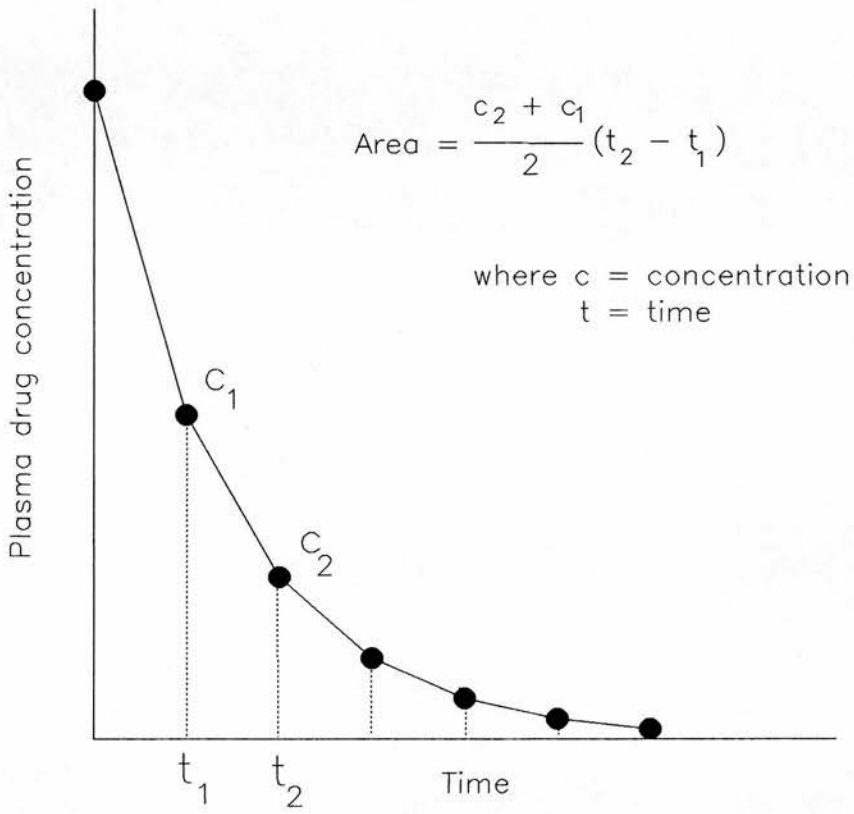

Figure 11 : Plasma drug concentration versus time curve can be divided into a series of trapezoids and the area under the curve can be calculated by the trapezoidal rule method.

The sum of the areas of all the trapezoids yields an estimate of $AUC_{0\text{-}20}$. The amount of unbound heparin in plasma in the first tertile after administration was ($AUC_{0\text{-}20\ Unbound}$), and the total heparin in plasma in the first tertile after administration was ($AUC_{0\text{-}20\ Total}$). ($AUC_{0\text{-}20\ Unbound}$) was expressed as a percentage of ($AUC_{0\text{-}20\ Total}$). As ($AUC_{0\text{-}20\ Total}$) was the same in control and endotoxaemic animals (for each heparin at each dose, $p > 0.1$ control v LPS-treated animals : Table 12), a reduction in ($AUC_{0\text{-}20\ Unbound}$) in endotoxaemic animals compared with control animals represents a reduction in the absolute amount of unbound heparin in endotoxaemic animals compared with controls.

Heparin dose (anti-factor Xa U/kg)		$AUC_{(0\text{-}20\ Total)}$ (U/ml. min) Control	LPS-treated	*p value*
UFH	**20**	**8.38 ± 0.87**	**8.21 ± 1.11**	**0.85**
	50	**14.23 ± 0.58**	**17.61 ± 2.65**	**0.24**
	100	**42.86 ± 3.40**	**47.48 ± 6.42**	**0.52**
LMWH	**20**	**6.33 ± 0.87**	**6.73 ± 0.51**	**0.70**
	50	**12.87 ± 1.49**	**13.64 ± 1.14**	**0.70**
	100	**27.74 ± 5.48**	**30.15 ± 3.77**	**0.74**

Table 12 : $AUC_{(0\text{-}20\ Total)}$ in control and LPS-treated animals. Results presented are the mean ± SEM of 5 experiments.

Heparin recovery and LAH in vivo

To assess the effect of RES blockade on plasma heparin concentrations, the anti-

factor Xa activity in plasma from control animals at 5, 10, 15 and 20 minutes after a bolus i.v. injection of 50 anti-factor Xa U/kg of heparin was compared with the anti-factor Xa activity in plasma at similar times in animals that received LAH in vivo 10 minutes prior to the bolus injection of heparin. In animals that received LAH as well as heparin, at each time point the anti-factor Xa activity of the excess LAH was subtracted from the plasma anti-factor Xa activity to give the anti-factor Xa activity of the heparin. The same comparison was made in animals that had been pretreated with LPS 2 hours prior to the heparin injection. Similarly, the plasma anti-factor Xa activity in control and LPS-treated animals that received LAH in vivo 15 minutes after a bolus i.v. injection of 50 anti-factor Xa U/kg of heparin was measured at various time points up to 1 hour after heparin administration. Again, at each time point after LAH administration, the plasma anti-factor Xa activity of heparin was calculated by subtracting the anti-factor Xa activity of the excess LAH from the plasma anti-factor Xa activity.

Statistical Analysis

The values are reported as mean ± SEM. Differences were determined with Student's *t*-test and were considered statistically significant when $p<0.05$ (Phillips, 1992b).

APPENDIX 1

CLEARANCE KINETICS : METHODOLOGY

Plasma radioactivity from total and free ^{125}I : methodology

For each time point, the amount of radioactivity in plasma from total and free ^{125}I was determined. The total amount of radioactivity in plasma was determined by counting the γ radiation emitted in one minute from100μL PPP in 900μL 0.85M saline, in duplicate. The amount of radioactivity from free ^{125}I in plasma was determined after ethanol precipitation of bound ^{125}I-heparin. Initial experiments determined that precipitation of bound ^{125}I-heparin was maximised by addition of 1 vol excess unlabelled heparin (1mg heparin/100μL 0.85M saline), 1 vol 0.85M saline and 9 vol absolute ethanol to 1 vol PPP. For each time point, 100μL PPP were added to 100μL 0.85M saline, 100μL excess unlabelled heparin (1mg heparin/100μL 0.85M saline) and 900μL 100% ethanol, in duplicate. To determine the amount of free ^{125}I in the injected mixture of heparin and radiolabelled tracer, 20μL of 2DS were added to 80μL normal rabbit serum (stock sample), 100μL 0.85M saline, 100μL excess unlabelled heparin (1mg hepairn/100μL 0.85M saline) and 900μL absolute ethanol, in duplicate. All samples (plasma, 2DS, ethanol precipitation) were kept at 4^0 C for 48 hours. The ethanol precipitation samples were then centrifuged (1600 x g for 30 minutes at room temperature) and 500μL supernatant were added to 500μL 0.85M saline. The γ radiation emitted in one minute from each plasma sample, the 10μL 2DS (see below) and each ethanol precipitation supernatant sample was counted in duplicate (gross CPM) and the mean gross CPM of the duplicate samples was calculated. The machine background radioactivity was calculated from the mean CPM of 4 x 1ml 0.85M saline samples; this value was subtracted from the mean gross

CPM of each sample, giving the CPM net of machine background radioactivity (net CPM).

At each time point, the percentage of the total injected dose of radioactivity present in plasma was calculated using the following methodology (Figures 12A-C). All weights were recorded using an electronic balance (Oertling, Avery Berkel, Canada).

FIGURE 12A

Each rabbit was assigned a unique identification number and its weight was recorded (**a**). The volume of radiolabelled tracer required to give a radioactivity dose of ~20.10^6 CPM (**b**) and the volume of stock solution containing the allocated dose of heparin (**c**) were recorded. The specific activities of the heparins were determined by the manufacturers (LMWH-100 anti-Xa U/mg; UFH- 160 anti-Xa U/mg)

Dose Injected

Before injection into the rabbit, the weight of the (heparin + radiolabelled tracer) mixture (in 5 ml volume syringe with sheathed 23G needle attached) was recorded (**d : Full Syringe**). Two drops of the mixture were added to 1ml 0.85 M saline (giving the two drop sample, or 2DS), and the weight of the (heparin + radiolabelled tracer) mixture minus 2 drops was recorded (**e : Minus 2 drops**) which was also the weight of the injected syringe (**e : Wt of Injected Syringe**). The weight of the 2 drops (**f : Wt of drops**) is (**d - e**). To allow the total injected dose of radioactivity to be calculated, 10 µL of the 2DS were added to 1 ml 0.85M saline, in duplicate (10µL 2DS).

After injection, the weight of the empty syringe (5 ml volume syringe with sheathed 23G needle attached) was recorded (**g**) and the weight of (heparin + radiolabelled tracer) mixture injected (**h : Dose Injected**) is (**e - g**).

At each time point, the weight of the 5ml volume syringe containing the whole blood/citrate sample to a total volume of 1.5ml was recorded (**i: Full Syringe of Blood**). The syringe was emptied and reweighed (**j : Empty Syringe**), and the weight of the blood/citrate sample (**k : Difference**) is (**i - j**).

The volumes used to estimate the amount of free ^{125}I by ethanol precipitation of bound ^{125} I were recorded (**Ethanol Precipitation**) and any sampling problems or difficulties with the rabbit were noted along with the bladder urinary volume (**Additional Comments**).

Figure 12B : Total Clearance

i)Total Dose Injected (cpm total) is the total (free and bound ^{125} I) amount of radioactivity injected and is calculated from :

$$\frac{\mathbf{e}\text{ (weight of dose injected)}}{\mathbf{f}\text{ (weight of 2 drops)}} \times \frac{\text{(CPM of 10µL 2DS x 100)}}{1}$$

As the 2 drops were placed in a 1ml volume, (CPM of 10µL 2DS x 100) gives the CPM in the 2 drops sample.

ii) CPM/kg is CPM injected per kilogramme rabbit $= \frac{\mathbf{i}\text{ (total dose injected)}}{\mathbf{a}\text{ (weight of rabbit)}}$

iii) CPM/ml bld is CPM injected per ml blood $= \frac{\mathbf{ii}}{58}$ *

* assuming 58ml blood vol/kg (Canadian Council on Animal Care, 1993a).

iv) CPM/ml plasma is CPM (bound and free ^{125} I) injected per ml plasma $= \frac{\mathbf{iii}}{0.58}$

assuming a haematocrit of 0.42 (Canadian Council on Animal Care, 1993a), and no radioactivity bound to red blood cells (Young and Hirsh, 1990).

v) Weight of blood + 0.2ml citrate : k.

vi) Weight of blood : (**v** - 0.2).

vii) Weight of plasma : (**vi** x 0.58), assuming a haematocrit of 0.42 (Canadian Council on Animal Care, 1993a).

viii) Weight of plasma + 0.2 ml citrate : (**vii** + 0.2).

ix) CPM/ml plasma + citrate : (net CPM of each sample x 10), as the radioactivity in 100µL volumes of plasma and citrate was counted.

x) CPM/ml plasma :

$$\frac{\textbf{viii}\ (\text{weight of plasma} + 0.2\ \text{ml citrate})}{\textbf{vii}\ (\text{weight of plasma})} \times \textbf{ix}\ (\text{CPM/ml plasma} + \text{citrate})$$

xi) % of total dose : $\frac{\textbf{x}\ (\text{cpm/ml plasma})}{\textbf{iv}\ (\text{total cpm/ml plasma})} \times 100$

Figure 12C : Corrected Clearance

Total radioactivity (from bound and free ^{125} I) corrected for free ^{125} I using ethanol precipitation data.

xii) CPM/ml plasma + citrate is the CPM from free^{125} I per ml plasma + citrate and is calculated from radioactivity values of ethanol precipitation supernatant samples :

$$\text{net CPM from free } ^{125}\text{I} \times \frac{1200}{500} \times 10\ *$$

* Correction factor of x 24 ([1200÷500]x10) is used because i) net CPM from free^{125}I is the radioactivity from free^{125} I in 500µL of 1200µL ethanol precipitation mixture,

and ii) 100µL of plasma was added to ethanol precipitation mixture.

xiii) CPM/ml plasma is CPM from free ^{125}I per ml plasma :

$$\frac{\textbf{viii (weight of plasma + 0.2 ml citrate)}}{\textbf{vii (weight of plasma)}} \times \textbf{xii (CPM/ml plasma + citrate)}$$

xiv) Corrected CPM/ml plasma is the radioactivity from heparin-bound ^{125}I per ml plasma : **x** (CPM [bound and free ^{125}I]/ml plasma) - **xiii** (CPM [free ^{125}I]/ml plasma)

xv) % Non-Precipitable is the percentage of total ^{125}I that is free, or non-precipitable, per ml plasma :

$$\frac{\textbf{xiii (CPM from free } ^{125}\textbf{I/ml plasma)}}{\textbf{x (CPM from bound and free } ^{125}\textbf{I/ml plasma)}} \times 100$$

xvi) Corrected % is the amount of bound ^{125}I per ml plasma expressed as a percentage of **iv** CPM (bound and free ^{125}I) injected per ml plasma corrected for free ^{125}I in the following way :

1. **iv** CPM (bound and free ^{125}I) injected/ml plasma corrected for free ^{125}I :

a) CPM from free ^{125}I in 20µL 2DS is calculated from net CPM 20µL 2DS :

$$\text{net CPM 20µL 2DS} \times \frac{1200}{500} \; *$$

* Correction factor of x 2.4 is required because 20µL of 2DS were added to ethanol precipitation solution of final volume 1200µL and (net CPM 20µL 2DS) is the radioactivity in 500µL of the 1200µL volume.

b) CPM (from bound and free ^{125}I) in 20µL 2DS : (net CPM 10µL 2DS x 2).

c) Percentage of total dose injected that is free ^{125}I (let this be **x**) :

$$\frac{\text{a) CPM from free } ^{125}\text{I in 20µL 2DS *}}{\text{b) CPM from bound and free } ^{125}\text{I in 20µL 2DS *}} \times 100$$

* as 20µL 2DS is representative of 2DS and so total injected dose.

d) CPM (free^{125} I) injected/ml plasma :

iv CPM (bound and free^{125} I) injected/ml plasma $_{x}$ $\frac{\mathbf{x}}{100}$

e) Corrected CPM (bound ^{125}I) injected/ml plasma :

iv CPM(bound and free^{125}I) injected/ml plasma - d) CPM(free^{125} I) injected/ml plasma

2. At each time point, amount of bound ^{125} I per ml plasma expressed as a percentage of the corrected CPM (bound ^{125} I) injected per ml plasma = **xvi** :

$$\frac{\textbf{xiv (CPM bound } ^{125}\text{I / ml plasma)}}{\text{e) corrected } \textbf{iv} \text{ (CPM bound } ^{125}\text{I injected / ml plasma}} \times 100$$

FIGURE 12A

Date _____

[a] **Rabbit #** RR#55

[b] **Volume of tracer** 125µL ≈ 21.10^6 CPM

Weight 3.0 kg

[C] **Volume of heparin** 1.5ml (0.94mg) of 12.5mg UFH in 20ml N saline → ~ 50anti-Xa U/kg

DOSE INJECTED: (g)

(g)

[d] **Full Syringe** 5.8489

[e] **Wt of Injected Syringe** 5.8261

[e] **Minus 2 drops** 5.8261

[g] **Empty Syringe** 4.3147

[f] **Wt of drops** 0.0228

[h] **Dose Injected** 1.5114

Time	[i] Full Syringe of Blood	[j] Empty Syringe	[k] Difference
1 min	5.1143	3.6174	1.4969
2 min	5.0608	3.5929	1.4679
3 min	5.0774	3.6045	1.4729
4 min	5.1014	3.6201	1.4813
5 min	5.0337	3.5431	1.4906
10 min	5.1168	3.6184	1.4984
15 min	5.0488	3.5394	1.5094
20 min	5.0408	3.5339	1.5069
25 min	5.0245	3.5222	1.5023
30 min	5.0422	3.5814	1.4608
40 min	5.0501	3.5309	1.5192
50 min	5.0258	3.5082	1.5176
60 min	5.0343	3.5308	1.5305

Ethanol Precipitation **Vol of plasma** 100µL + 100µL UFH (1 mg UFH/ 100µL 0.85M saline)

Vol of 0.85 M NaCl 100µL

Vol of absolute ethanol 200µL

Additional Comments Bladder urinary volume : 15ml.

FIGURE 12B : Total Clearance

i) Total Dose Injected = 12,256,923 cpm total = ii) 4,085,641 cpm/kg

= iii) 70,443 cpm/ml blood = iv) 121,454 cpm/ml plasma

(assuming 58ml blood vol/kg; 0.58 plasma ratio)

Free ^{125}I 20μL 2DS = (269 x 2.4) = 646
Total ^{125}I 20μL 2DS = (2 x 1849) = 3698
∴ 17.5% non-precipitable / free ^{125}I
Corrected CPM/ml plasma = (121.454 - 21,255)
= 100,199

	(v)	(vi)	(vii)	(viii)	(ix)	(x)	(xi)
Time	Wt of blood + 0.2ml citrate	Wt of blood (-0.2ml)	Wt of plasma (x 0.58)	Wt of plasma + 0.2ml citrate	cpm/ml plasma + citrate	cpm/ml plasma (Total ^{125}I)	% of Total Dose
1 min	1.4969	1.2969	0.7522	0.9522	44890	56826	46.8
2 min	1.4679	1.2679	0.7354	0.9354	37650	47890	39.4
3 min	1.4729	1.2729	0.7383	0.9383	35280	44838	36.9
4 min	1.4813	1.2813	0.7432	0.9432	34370	43620	35.9
5 min	1.4906	1.2906	0.7485	0.9485	31710	40183	33.1
10 min	1.4984	1.2984	0.7531	0.9531	24870	31475	25.9
15 min	1.5094	1.3094	0.7595	0.9595	19540	24686	20.3
20 min	1.5069	1.3069	0.7580	0.9580	17040	21537	17.7
25 min	1.5023	1.3023	0.7553	0.9553	16720	21148	17.4
30 min	1.4608	1.2608	0.7313	0.9313	14520	18492	15.2
40 min	1.5192	1.3192	0.7651	0.9651	11480	14481	11.9
50 min	1.5176	1.3176	0.7642	0.9642	9680	12214	10.1
60 min	1.5035	1.3035	0.7560	0.9560	8930	11293	9.3

FIGURE 12C : Corrected Clearance (EtOH Precipitation)

	(xii)	(xiii)	(xiv)	(xv)	(xvi)
Time	cpm/ml plasma + citrate	cpm/ml plasma	corrected cpm/ml plasma	% Non-Precipitable	Corrected %
1 min	6220	7874	48952	13.9	48.9
2 min	5380	6844	41046	14.3	41.0
3 min	4880	6202	38636	13.8	38.6
4 min	4710	5978	37642	13.7	37.6
5 min	3990	5057	35126	12.6	35.1
10 min	2880	3645	27830	11.6	27.8
15 min	2760	3487	21199	14.1	21.1
20 min	1880	2377	19160	11.0	19.1
25 min	2160	2732	18416	12.9	18.4
30 min	1900	2420	16072	13.1	16.0
40 min	1370	1729	12752	11.9	12.7
50 min	1230	1552	10662	12.7	10.6
60 min	1300	1644	10649	14.6	10.6

Pharmacokinetics

For each animal, the percentage of the injected dose of radioactivity present in 1ml plasma (% corrected) was plotted against time after transformation of radioactivity into logarithmic values. The curvilinear profile of the semilogarithmic plasma concentration versus time curve suggested a 2-compartment model where the biexponential curve can be described by 2 exponential terms; the slope of the residual line is $-\alpha$ where α is the distribution rate constant of the drug, while the terminal slope from the post-distributive phase of the curve is $-\beta$ where β is the terminal elimination rate constant of the drug. The distribution and elimination half-lives ($t_{1/2}\,\alpha$ and $t_{1/2}\,\beta$) were determined by linear regression analysis. The slope of the regression line representing the distribution phase is $-\alpha$ and $t_{1/2}\,\alpha = 0.693/\alpha$; the slope of the regression line representing the elimination phase is $-\beta$ and $t_{1/2}\,\beta = 0.693/\beta$, according to the following (Gillies et al, 1986) :

all straight lines can be defined by :

$$\mathbf{Y = mX + b}$$

where : **m** = the slope of the line and **b** = intercept of the Y axis.

For semilogarithmic plasma drug concentration versus time plots :

Y = ln (natural log) of drug concentration in plasma
X = time after dose
m = slope of line
b = intercept on ln plasma drug concentration axis

giving :

ln drug concentration = (slope x time) + ln concentration at Y intercept

Where drug elimination is first-order :

slope = - elimination rate constant

or : **- slope = elimination rate constant**

Therefore, Y = mX + b becomes :

ln drug concentration = (- elimination rate x time) + ln y-intercept

Let :

ln C = ln of drug concentration
K = elimination rate constant
t = time after dose
ln C_o = ln of drug concentration at intercept of y axis

giving :

$$\ln C = \ln C_o - K_t$$

By definition of half-life ($t_{1/2}$), the concentration (**C**) at the time (**t**) equal to the half-life is one-half of the original concentration (C_o). Therefore, at one $t_{1/2}$, the concentration is one-half of what it was at the start, or at $t = t_{1/2}$, $C = 1/2\ C_o$. Assuming $C_o = 1$, then :

$$\ln 0.5\ C_0 = \ln C_0 - K(t_{1/2})$$

$$\rightarrow \ln 0.5 = \ln 1 - K(t_{1/2})$$

Transforming this equation algebraically gives :

$$K(t_{1/2}) = \ln 1 - \ln 1/2$$

$$t_{1/2} = \frac{0 - (- 0.693)}{K}$$

$$t_{1/2} = \frac{0.693}{K}$$

As α and β are similar to K in that they represent the distribution and terminal elimination rate constants, respectively, it follows that :

$$t_{1/2} = \frac{0.693}{\alpha} \quad \text{and} \quad t_{1/2} = \frac{0.693}{\beta}$$

where :

α : negative slope of the residual line
β : negative slope of the line from the post-distributive phase of the curve

and slope of the line is :

$$\frac{\ln C_2 - \ln C_1}{t_2 - t_1}$$

where :

C_2 : second concentration
C_1 : first concentration
t_2 : time corresponding to C_2
t_1 : time corresponding to C_1

APPENDIX 2

RABBIT ORGAN WEIGHTS

Weight of Organs in 2.5kg male New Zealand White rabbit

Organ [1]	Container and Tissue [2] (g)	Container (g)	Tissue (g)	Total Organ (g)
Liver [3]	33.8083	14.4710	19.3373	
	33.5180	14.1506	19.3674	
	33.5012	14.2165	19.2847	73.1399
	28.8123	13.6618	15.1505	
Spleen	14.3454	13.5478	0.7976	0.7976
R kidney	20.6102	13.3535	7.2567	14.7231[4]
L kidney	20.9163	13.4499	7.4664	

[1] Organs from 2.5 kg rabbit used for assessment of in vivo anticoagulant activity of excess LAH were removed intact at post mortem, blotted to dryness and placed in polypropylene containers prior to weighing on electronic scales.

[2] The weight of the container and tissue was recorded, the tissue was removed, and the empty container was weighed. The weight of the tissue is the difference in weights between the full and empty container.

[3] The liver had to be divided into 4 pieces to facilitate removal as it was impractical to remove it whole.

[4] The weights of the right (**R**) and left (**L**) kidneys were added to give the weight of renal tissue in the rabbit.

APPENDIX 3

PUBLICATIONS

The variable anticoagulant response to unfractionated heparin in vivo reflects binding to plasma proteins rather than clearance

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The anticoagulant response to fixed doses of unfractionated heparin is variable in patients with acute illness, and some patients with venous thromboembolism require high doses of heparin to achieve a therapeutic anticoagulant response. To investigate the mechanism responsible for the variable anticoagulant response to heparin in acute illness, heparin clearance and nonspecific protein binding were compared in control and endotoxin-treated rabbits. The plasma half-life ($t_{1/2}$) of radiolabeled heparin increased in a dose-dependent fashion. At all doses of heparin studied, the $t_{1/2}$ of radiolabeled heparin was unaffected by experimental endotoxemia when compared with control animals. In contrast, the amount of heparin recovered was lower in the plasma of endotoxemic animals because of increased binding to plasma proteins. A chemically modified heparin with low affinity for antithrombin III was added ex vivo or in vivo to displace heparin bound nonspecifically to plasma proteins. The proportion of heparin bound to plasma proteins was significantly greater in the plasma of endotoxemic animals than in controls. These findings indicate that acute inflammation alters heparin recovery but does not affect heparin clearance. The variability of the anticoagulant response to heparin seen in patients with thromboembolism may, in part, be due to this effect of the underlying disease process. (J Lab Clin Med 1997;130:649-55)

Abbreviations: AUC = area under the curve; LAH = low-affinity heparin; LPS = lipopolysaccharide; $t_{1/2}$ = plasma half-life; SHPP = 3-(4-hydroxyphenyl) propionic acid N-hydroxysuccinimide ester

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Heparin is an effective anticoagulant in a variety of thrombotic disorders.[1] The use of heparin can be complicated by marked variability in the dose response among patients with thrombosis.[2] In addition, some patients with acute thrombosis require very high doses of heparin to obtain an adequate response.[3]

The anticoagulant effect of heparin is mediated through its specific binding to antithrombin III.[4-6] Heparin also binds nonspecifically to a number of plasma proteins such as platelet factor-4, histidine-rich glycoprotein, vitronectin, fibronectin, and von Willebrand factor.[7] There is evidence that these

nonspecific interactions can reversibly neutralize the anticoagulant effect of heparin by limiting the amount of heparin available to bind to antithrombin III.[8,9] Some of the heparin-binding proteins are acute phase reaction proteins, the levels of which increase to a variable extent during illness.[10-12]

There are two potential explanations for the marked variability of the anticoagulant response to heparin. First, nonspecific binding of heparin to plasma proteins[8,9] or to platelet factor-4[13,14] or high-molecular-weight von Willebrand factor,[15] which are released from activated platelets or endothelial cells, may reduce the amount of heparin available to catalyze antithrombin III. In previous studies, we showed that nonspecific binding of unfractionated heparin to plasma proteins markedly impairs its anticoagulant activity in patients with venous thromboembolism.[16] A second possibility is that alterations in heparin clearance may be involved. It is generally accepted that unfractionated heparin is cleared from the blood by a combination of a saturable mechanism and a nonsaturable mechanism.[17,18] The saturable mechanism is thought to reflect heparin binding to vascular endothelial cells and reticuloendothelial cells. In support of these concepts, cultured endothelial cells have specific heparin binding sites on their surface,[19,20] and these cells as well as macrophages have been shown to internalize and degrade heparin.[21-23] At low doses, heparin is cleared mainly by the highly efficient saturable mechanism. At higher doses, the cellular binding sites are saturated, and heparin is cleared predominantly by renal elimination.[17,18] Thus any alterations in heparin clearance caused by increased binding to endothelial cells or blockade of the reticuloendothelial system may explain the reduced heparin recovery in acutely ill patients.

The purpose of this study was to examine the mechanism responsible for reduced heparin recovery in disease states. To accomplish this, we used endotoxin infusion to induce acute illness in rabbits and examined the relative roles of reversible, nonspecific protein binding and heparin clearance as determinants of heparin recovery.

METHODS

Reagents. Unfractionated heparin (160 anti-factor Xa U/mg) was obtained from Rhône-Poulenc Rorer, Montreal, Canada, or from Sigma Chemical Co., St. Louis, Mo. (157 anti-factor Xa U/mg). SHPP was supplied by Pierce, Brockville, Canada. Carrier-free iodine 125 was obtained from Dupont, Wilmington, Del. Purified bacterial LPS obtained from *Salmonella minnesota* R595 (Sigma Chemical Co.) was suspended in sterile phosphate-buffered saline solution at a concentration of 2 mg/ml and was solubilized by sonication.

Preparation of ^{125}I-labeled heparin Unfractionated heparin was reacted with SHPP and labeled with ^{125}I according to the method of Dawes and Pepper.[24] When compared with the unlabeled starting material, the molecular weight distribution (as determined by high-performance liquid chromatography)[9] and biologic activity (as measured by anti-factor Xa assay) of the ^{125}I-labeled heparin were unchanged.

Preparation of LAH. LAH was prepared from unfractionated heparin by controlled periodate oxidation and borohydride reduction according to the method of Casu et al.[25] with minor modifications[26] to remove residual high-affinity material. The anti-factor Xa activity of the LAH was <1.0 U/mg.

Heparin recovery and clearance in healthy and LPS-treated rabbits. All animal studies were carried out in accordance with the recommendations of the Canadian Council of Animal Care and were approved by the Animal Research Ethics Board of McMaster University.

Specific pathogen-free New Zealand White male rabbits (Charles River Laboratories, St. Constant, Canada), ranging in weight from 2.5 to 3.0 kg were given a 1.5 ml bolus of either LPS (10 μg/kg diluted in 0.9% NaCl) or an equivalent volume of saline solution into the marginal ear vein. Two hours later the rabbits received a 1.5 ml bolus of either 20, 50, or 100 anti-factor Xa U/kg of heparin to which a tracer amount (10 μCi, <1 anti-factor Xa U) of ^{125}I-labeled heparin had been added. The heparin doses chosen span the doses used clinically. In addition, these doses have been shown to have been cleared predominantly by the saturable mechanism of heparin clearance.[18] The total radioactivity administered was determined in a gamma counter (model 1272; LKB-Pharmacia, Fisher Scientific, Toronto, Canada) before injection. In some experiments, LPS-treated or control rabbits were given a bolus of LAH (6.25 mg/kg, 20-fold molar excess) either 10 minutes before or 15 minutes after receiving 50 anti-factor Xa U/kg of heparin. LAH was used to displace anticoagulantly active heparin from plasma proteins other than antithrombin III, to displace heparin bound to endothelial cells, and to block uptake by the reticuloendothelial cells. To study the effect of LAH alone, two control rabbits were given only a bolus of LAH.

Preparation of platelet-poor plasma. Blood samples (1.3 ml into 200 μl of 3.8% sodium citrate) were taken from the contralateral central ear artery immediately before and at 5-minute intervals after heparin administration. The blood lost was replaced with normal saline solution. The experiments were terminated at the end of 1 hour and the rabbits were killed. Each blood sample was carefully weighed to determine the mass of blood collected. After centrifugation at 1600 *g* at room temperature for 15 minutes, platelet-poor plasma was harvested.

Heparin recovery based on anticoagulant activity. To monitor heparin recovery, the anti-factor Xa activity of heparin in each plasma sample was determined by using

Table I. Recovery of various doses of heparin from the plasma of control and LPS-treated rabbits

Dose (anti-Xa U/kg)	(1) Amount recovered (U × ml^{-1} × min)	(2) Amount recovered after LAH addition (U × ml^{-1} × min)	(3) Amount bound to plasma proteins (U × ml^{-1} × min)	(4) Percent bound to plasma proteins
Control				
20	5.0 ± 0.6	8.4 ± 1.0	3.4 ± 0.7	39.6 ± 4.5*
50	9.5 ± 0.6	14.2 ± 0.6	4.8 ± 0.4	33.7 ± 2.7†
100	32.9 ± 1.9	42.9 ± 3.4	10.0 ± 2.3	22.4 ± 3.7†
LPS-treated				
20	3.5 ± 0.7	8.1 ± 1.1	4.6 ± 0.7	56.8 ± 6.7*
50	10.0 ± 1.5	17.6 ± 2.7	7.6 ± 1.2	43.4 ± 1.8†
100	28.9 ± 2.1	47.5 ± 6.4	18.4 ± 4.7	37.2 ± 5.5†

Values are expressed as mean ± SEM for 4 or 5 animals per group. The amount of heparin recovered was determined as anti-factor Xa activity and was calculated from the AUC for the first 20 minutes after administration of heparin (column 1). Excess LAH was added ex vivo to displace heparin from plasma protein-binding sites, and the AUC again was calculated (column 2). The difference between column 1 and column 2 is the amount bound to plasma proteins (column 3) and is used to determine the percentage of heparin bound to plasma proteins (column 4).
*$p = 0.8$.
†$p < 0.05$.

the method of Teien and Lie.[27] The Strachrom heparin kit from Diagnostica Stago (Wellmark Diagnostics, Guelph, Canada) was adapted for use on the ACL automated coagulation analyzer (Instrumentation Laboratory, Toronto, Canada). Aliquots of pooled platelet-poor plasma from healthy rabbits to which known amounts of heparin were added were used as standards.

To assess the amount of heparin that is bound nonspecifically to plasma proteins (reversible heparin binding) ex vivo, the anti-factor Xa activity was measured before and after the addition of 90 μg/ml of LAH. This concentration of LAH was chosen because previous studies demonstrated that it exceeds the amount of LAH needed to totally displace the protein-bound heparin.[8] The anti-factor Xa activity measured before LAH addition reflects the amount of heparin that is not bound to plasma proteins. By subtracting this value from the anti-factor Xa activity measured after LAH addition, the amount of heparin bound to plasma proteins can be calculated.

Recovery of heparin in the first 20 minutes after heparin injection was calculated by plotting the heparin concentration as a function of time and measuring the AUC by using the trapezoidal rule method.[28] We restricted analysis to the first 20 minutes after heparin administration, because at the heparin doses tested, most of the anti-factor Xa activity was cleared during this time interval.

Clearance of ^{125}I-labeled heparin. To determine heparin clearance, two 100 μl aliquots of plasma samples from each time point were each mixed with 300 μl of absolute alcohol to precipitate heparin and plasma proteins. After incubation overnight at 4° C, the samples were centrifugated at 4000 *g* for 15 minutes at room temperature, and the amount of ^{125}I-labeled heparin in the precipitate was determined in a gamma counter. The disappearance of labeled heparin as a function of time was then plotted, and the $t_{1/2}$ was determined by linear regression analysis after logarithmic transformation. The slope of the regression line, which represents the elimination phase, is designated -β, and the $t_{1/2}$ is $0.693/\beta$.[28]

Statistical analysis. The values are reported as mean ± SEM. All data were analyzed by using Student's *t* test, and differences were considered significant at a value of $p < 0.05$.

RESULTS

Recovery of heparin ex vivo. Anti-factor Xa activity in each plasma sample was assayed before and after the addition of 90 μg/ml of LAH. Because LAH displaces anticoagulantly active heparin from plasma proteins, the anti-factor Xa activity after LAH addition represents the total amount of heparin recovered. This was determined from the AUC for the first 20 minutes after heparin administration.

The amount of heparin that bound nonspecifically to plasma proteins was calculated by subtracting the amount recovered before LAH addition from that measured after LAH addition. As shown in Table I, the amount of heparin bound to plasma proteins is markedly higher in the LPS-treated group than in controls for each dose of heparin administered. Thus the percentage of heparin bound nonspecifically to plasma proteins is significantly higher (approximately 1.5-fold, $p < 0.05$) in the LPS-treated animals who received 50 and 100 anti-factor Xa U/kg when they are compared with controls, and the result is close to significance ($p = 0.08$) in the group that received 20 anti-factor Xa U/kg.

Comparison of unfractionated heparin recovery ex vivo and in vivo. To compare the amount of heparin that could be recovered when LAH is added ex vivo

Table II. Amount of heparin recovered when LAH is added ex vivo or is administered in vivo

	Amount recovered after LAH ($\frac{U}{ml} \times$ min)	
	LAH added ex vivo*	LAH administered in vivo†
Control	14.2 ± 0.6	15.5 ± 0.7
LPS-treated	17.6 ± 2.7	18.2 ± 1.4

All rabbits received 50 anti-Xa U/kg of heparin. The amount of heparin recovered was determined as anti-factor Xa activity and was calculated from the AUC for the first 20 minutes after administration of heparin. Values are expressed as mean ± SEM for 4 or 5 animals/group. There are no significant differences between the groups ($p > 0.1$).

*Excess LAH was added to each sample ex vivo.

†Excess LAH was administered in vivo 10 minutes before injection of heparin.

Table III. Plasma $t_{1/2}$ values of ^{125}I-labeled heparin in control and LPS-treated animals after intravenous injection of various doses

Dose (anti-Xa U/kg)	Plasma $t_{1/2}$ (min)		
	Control	LPS-treated	*p* value
20	10.0 ± 1.0	11.9 ± 1.1	0.28
50	13.6 ± 1.2	16.8 ± 0.9	0.07
100	22.5 ± 0.7	23.6 ± 1.9	0.60

Plasma $t_{1/2}$ values were determined by linear regression analysis of the semilogarithmic plot of plasma radioactivity versus time. The slope of the regression line representing the elimination phase is $-\beta$, and the $t_{1/2}$ is $0.693/\beta$. Values are expressed as mean ± SEM for 4 or 5 animals/group.

(see above) with the recovery of heparin when LAH is given in vivo, excess LAH (6.25 mg/kg) was administered 10 minutes before the injection of heparin (50 anti-factor Xa U/kg). As a control, some rabbits were given LAH alone, and the small amount of anti-factor Xa activity, when measured in the plasma of these rabbits, was subtracted from the anti-factor Xa activity measured in the plasma of rabbits that received heparin after being given LAH. The amount of heparin recovered was then determined in terms of anti-factor Xa activity and expressed as the AUC for the first 20 minutes after heparin injection.

As shown in Table II, there is no significant difference between the total amount of heparin that can be recovered when LAH is added ex vivo or when LAH is injected in vivo. Furthermore, there is no significant difference in heparin recovery between the LPS-treated and the control animals. These results show that LAH is able to displace heparin from protein binding sites in vivo and that the extent of reversible heparin neutralization that occurs in vivo can be assessed reliably ex vivo by adding LAH.

Clearance of ^{125}I-labeled heparin. Tracer amounts of ^{125}I-labeled heparin were added to various doses of unlabeled heparin and injected into control or LPS-treated animals. The $t_{1/2}$ values were calculated from the radioactivity in the plasma samples and are shown in Table III. The elimination of ^{125}I-labeled heparin was dose-dependent and increased as the dose of unlabeled heparin increased. Thus the $t_{1/2}$ after administration of 100 anti-factor Xa U/kg was 2.2 times longer than after 20 anti-factor Xa U/kg and was 1.7 times longer than after 50 anti-factor Xa U/kg. However, there is no significant difference in the $t_{1/2}$ of ^{125}I-labeled heparin in LPS-treated rabbits when compared with that in controls at each dose of unlabeled heparin administered.

Clearance of radioactivity and anti-factor Xa activity after LAH in vivo. Further experiments were performed to determine whether LAH administration in vivo displaced heparin bound to endothelial cells in addition to displacing heparin bound to plasma proteins. To achieve this aim, heparin (50 anti-factor Xa U/kg) and tracer amounts of radiolabeled heparin were given to a group of LPS-treated rabbits, and this was followed 15 minutes later by LAH (6.25 mg/kg). If heparin was also bound to vascular endothelial cells, the addition of LAH would be expected to increase both the anti-factor Xa activity (because of heparin bound to plasma proteins) and radioactivity (because of heparin bound to vascular endothelium).

As shown in Fig. 1, when LAH was administered 15 minutes after heparin, there was an almost 50% increase in anti-factor Xa activity (from 0.39 ± 0.03 U/ml immediately before LAH administration to 0.58 ± 0.06 U/ml 5 minutes after LAH administration; $p < 0.02$). In contrast, there was no significant increase in the amount of radiolabeled heparin recovered in the plasma (i.e., 28.0% ± 4.5% of the total radiolabeled dose injected was present immediately before LAH administration and 26.4% ± 3.6% of the total radiolabeled dose injected was present 5 minutes later; $p > 0.05$). These findings indicate that at the dose of heparin studied, the increase in anticoagulant activity in plasma after administration of LAH in vivo is the result of displacement of heparin from circulating heparin-binding proteins and is not caused by displacement of heparin from endothelial binding sites. There was, however, a change in the slope of the radioactivity clearance curve after LAH administration. Because the clearance of unfractionated heparin is dose dependent (Table III),[17,18] this change in slope likely

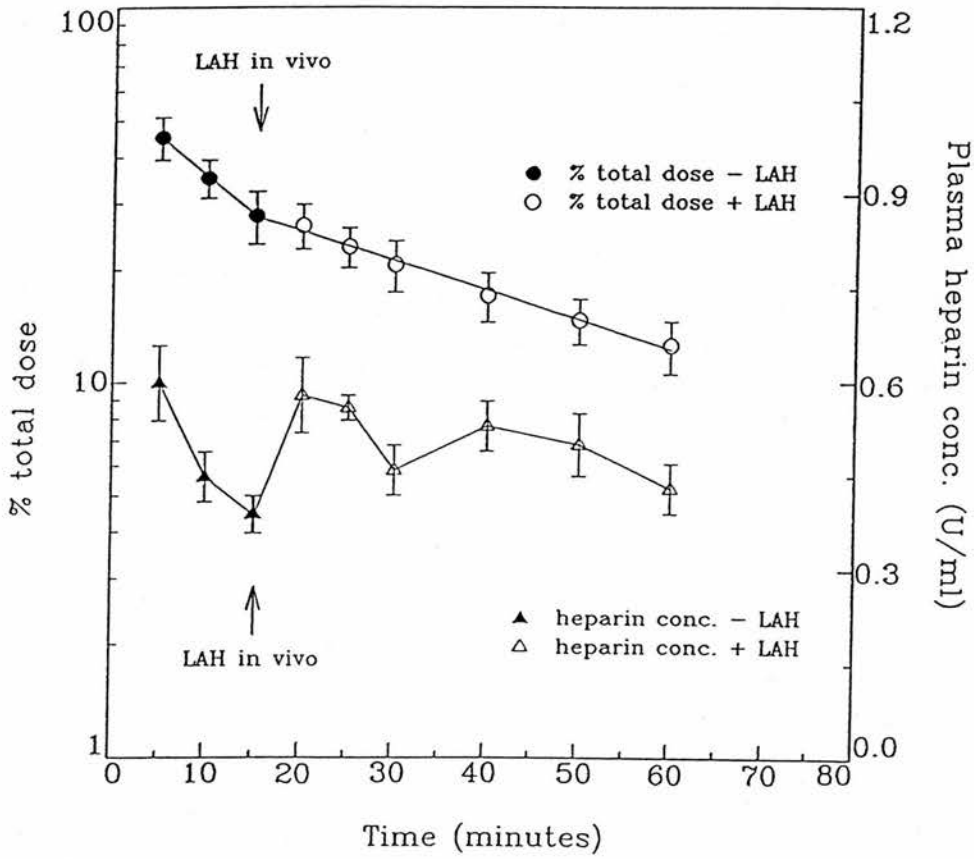

Fig. 1. Effect of LAH administered in vivo on the clearance of plasma heparin concentration and radioactivity in LPS-treated rabbits. Heparin (50 anti-factor Xa U/kg) mixed with tracer amounts of radiolabeled heparin was administered first, followed 15 minutes later by the injection of LAH (6.25 mg/kg) to displace heparin from protein-binding sites. Plasma heparin concentrations (measured as anti-factor Xa activity) and radioactivity were measured as described in Methods. LPS was administered 2 hours before the heparin. Values are expressed as mean ± SEM for 4 animals.

reflects the increase in heparin concentration caused by LAH administration.

DISCUSSION

The relative contributions of nonspecific plasma protein binding and altered clearance to the variability in dose response to heparin and to the impaired anticoagulant response observed in some patients with acute venous thromboembolism are uncertain. Our group has reported that heparin binding to plasma proteins is an important determinant of the heparin dose required to produce a therapeutic heparin level.[16] The results of our study suggested that increased levels of heparin-binding proteins contribute to heparin resistance observed among patients with venous thromboembolism but that other mechanisms may also play a role in the reduced heparin recovery observed in patients with high heparin requirements.[29]

In this study we examined the effect of acute inflammation on heparin clearance and reversible protein binding. We used an established experimental model (endotoxin administration) to induce acute illness in rabbits. This model has been shown to increase the level of heparin-binding proteins in rat plasma[30] and to potentiate the development of venous thrombosis in rabbits.[31]

Our results show that the amount of heparin that is bound nonspecifically to plasma proteins is markedly elevated in the plasma of LPS-treated rabbits (Table I). This is similar to the increase observed in LPS-treated rats.[30] We attribute these findings to an increase in the plasma levels of heparin-binding proteins. Moreover, our results show that the increase in protein binding, which was previously demonstrated by the addition of LAH in vitro[8,16] or ex vivo,[16,32] can also be demonstrated in vivo (Table II).

We used a dose of endotoxin that produced major multisystem perturbations within 2 hours of administration.[33] Because hepatic acute-phase protein mRNA does not increase significantly for 4 to 8 hours after acute phase induction, and because the release of newly synthesized acute phase proteins occurs many hours after the onset of inflammation,[34-36] it is likely that the model of acute inflammation used in our studies predominantly assessed the extent of reversible heparin binding to proteins that are released from storage sites in endothelial and other vascular cells as a response to endotoxin injury. Thus heparin-binding proteins such as platelet-factor 4,[13,14] lactoferrin,[37] and high-molecular-weight multimers of von Willebrand factor[15] may be released from endotoxin-activated platelets, neutrophils, and endothelial cells, respectively. A similar situation may occur in human patients, because patients with thrombotic disorders can show a reduced anticoagulant response to heparin within a few hours of onset of their symptoms.

Our results demonstrate that reversible heparin binding is an important mechanism for the reduced heparin recovery in acute illness. In contrast, we found no evidence of increased heparin clearance. Thus the circulating $t_{1/2}$ of heparin in endotoxemic rabbits was not different from that in controls, even though heparin recovery was markedly reduced (Table III). Our finding that endotoxemia does not increase heparin clearance is similar to the previous observation that acute venous thrombosis does not reduce heparin $t_{1/2}$.[2] In contrast, heparin clearance has been reported to be increased in clinical and experimental pulmonary emboli.[38] This difference may be due to the nature of the thrombi found in venous thrombosis and in pulmonary embolism.

When LAH was given after heparin, it produced a marked increase in anticoagulant activity by displacing heparin from plasma proteins. In contrast, there was no change in the radioactivity clearance curve (Fig. 1). There are two potential explanations for these findings. First, it is possible that when given in clinical doses (i.e., 50 anti-factor Xa U/kg) heparin does not exhibit reversible binding to vascular endothelial cells or macrophages in the reticuloendothelial system. A second possibility is that heparin is rapidly internalized by these cells and thus inaccessible to displacement by LAH. This is supported by studies demonstrating that endothelial cells and macrophages can internalize and degrade heparin.[20-23,39]

In summary, using an animal model of acute endotoxemia, we have demonstrated that the phenomenon of impaired heparin recovery is caused by an increase in the concentration of heparin-binding proteins. This observation has important clinical implications, because nonspecific binding of heparin to plasma proteins decreases its anticoagulant effect by limiting the amount available to bind to antithrombin III. We found no evidence that increased heparin clearance contributes to the reduced heparin recovery observed in acute illness. Although the results in rabbits may be quantitatively different from those in human patients, they are likely to be qualitatively similar. Our studies suggest that the variable anticoagulant response to heparin seen in patients with venous thrombosis is likely to reflect plasma protein binding rather than alterations in heparin clearance.

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